REMARKS

1. Claims

Claims 1-9, 13, 15, 18-25, 58, 97, 125, and 262-300 are pending in the application, prior to entry of the present amendment. Claims 10-12, 14, 16-17, 26-57, 59-96, 98-124, and 126-261 were previously cancelled. Claims 1, 2, 4, 6-9, 18-19, 125, 262-265, 294-297 are currently amended. New claims 301-307 are added with the present amendment.

Claims 1, 2, 4, 6, 7, 8, 9, 262, 263, 264, and 265 have been amended through insertion of the term "subject to mutation" to clarify and differentiate the gene subject to mutation, and the cytidine deaminase (AID) gene. Support is found, for example, at [0125] in US 2005/0095712A1.

Claim 13 has been amended by insertion of the term "at the 5′ end". Support is found, for example, at [0112] in US 2005/0095712A1.

Claims 18 and 19 have been amended by correcting their claim dependency to those claims currently pending in the instant application.

Claim 97 has been amended by insertion of the term "wherein the cell is a myeloma". Support is found, for example, at [0146] in US 2005/0095712A1.

Claims 125, 294, 295, 296 and 297 have been amended by insertion of the term "first" to clarify references to the first and second antigens respectively in these claims. The amendment is supported throughout the instant specification.

New claims 301-303 relate generally to the additional step of separating the clonal colonies that comprise a mutation in the gene of interest, from the rest of the cells. Support is found, for example, at [0138] in US 2005/0095712A1.

New claims 304-306 relate generally to methods, wherein the cell is a non-B cell. Support is found, for example, at [0146] in US 2005/0095712A1.

New claim 307 refers to the method of claim 97, wherein the cell is a hybridoma. Support is found, for example, at [0102] in US 2005/0095712A1.

Based on the following remarks, Applicants respectfully request that the Examiner reconsider the outstanding rejection and that it be withdrawn.

2. OATH / DECLARATION

A new Declaration will be provided shortly.

3. CLAIM OBJECTIONS

The Examiner objects to claims 18 and 19 based on the informalities that these claims are partially dependent on claims that have been cancelled, and lack a hyphen between the numbers 1 and 17. These objections should be moot in view of the claim amendments above.

4. CLAIM REJECTIONS 35 USC §112 SECOND ¶

The Examiner rejects claims 6-9, 262-265, 294-297 and 299-300 as allegedly "being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." In particular, the Examiner is of the opinion that:

1. Claims 6-9 refer to the amount of poly A mRNA of "the gene" of the cell referred to in Claim 1, whereas Claim 1 has two distinct genes; the gene into which a mutation is being induced, and a transgenic AID gene. The Examiner concludes that "a skilled artisan would be unable to determine the meets and bounds of the claimed invention."

2. Claims 262-265 refer to the method of claim 1, wherein "the gene" of the cell referred to in claim 1, whereas Claim 1 has two distinct genes; the gene into which a mutation is being induced, and a transgenic AID gene. The Examiner concludes that "a skilled artisan would be unable to determine the meets and bounds of the claimed invention."

3. Claims 294-297 (and dependent claim 299 and 300) recite a change in affinity or specificity in a mutated antibody for "the antigen" of the antibody of the methodology of claim 125. Claim 125 recites a method of altering an affinity or a specificity of a monoclonal antibody to a second antigen. The Examiner concludes that "It is unclear to which antigen claims 294-297 are directed towards. If the method requires identification of the monoclonal antibody, one must be aware of where the mutations are directed towards. Mutations that effect affinity or specificity are not necessarily distinct from

mutations that alter cross-reactivity." Thus the Examiner contends a skilled artisan would be unable to determine the metes and bounds of the claimed invention.

It is believed that the rejections to the claims should be moot in view of the claim amendments above. Accordingly, reconsider and withdrawal of this rejection is respectfully requested.

5. CLAIM REJECTIONS UNDER 35 USC §103

A. Claims 1-4, 6-9, 13, 15, 19-22, 24-25, 58, 97, 125, 262-272, 276-284, 287-288-291, and 293-300 are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Wabl et al., (US Patent No. 5,885,827) in view of Muramatsu et al. (Class Switch Recombination and Hypermutation require Activation-Induced Cytidine Deaminase (AID) a Potential RNA Editing Enzyme, Cell, 2000 102 553-563). Applicants respectfully traverse the Examiner's assertion that the claimed invention would have been obvious to one of ordinary skill in the art at the time it was made.

The pending claims are generally directed to methods of inducing and identifying a mutation in a gene of interest (i.e. the method (i) below); or an antibody gene (method ii, below), as a system for changing the affinity or specificity of an antibody (method iii below); or for inducing class switch recombination in an antibody gene (method iv, below):

i) Independent claim 1

A method of inducing and identifying a mutation in a gene wherein the gene is operably linked to within about two kilobases of a promoter. The method comprising
expressing a transgenic activation induced cytidine deaminase (AID) gene in the cell and expressing the gene in the cell, and then subsequently establishing and culturing clonal colonies of the cell, and then identifying one or more clonal colonies that comprise a mutation in the gene subject to mutation.

ii) Independent claim 58

A method of inducing and identifying a mutation in an antibody gene in a eukaryotic cell. The method comprising, expressing a transgenic AID gene in the cell and expressing the antibody gene in the cell, establishing and culturing clonal colonies of the cell, and identifying one or more clonal colonies that comprise a mutation in the antibody gene.

iii) Independent claim 125

A method of altering an affinity or a specificity of a monoclonal antibody to a first antigen, or altering a cross reactivity of the monoclonal antibody to a second antigen, wherein the monoclonal antibody is produced by a eukaryotic cell, and wherein the cell is capable of expressing a transgenic AID gene under inducible control. The method comprising,

(a) expressing the AID gene in the eukaryotic cell for a time and under conditions sufficient to induce a mutation in a gene encoding the monoclonal antibody;

(b) suppressing expression of AID gene in the eukaryotic cell;

(c) establishing clonal colonies of the cell; and

(d) determining whether the monoclonal antibody produced by any of the clonal colonies of the cell has altered affinity or specificity to the first antigen, or altered cross reactivity to the second antigen.

iv) Independent claim 97

A method of inducing and identifying a class switch in an antibody heavy chain gene in a eukaryotic cell. The method comprising expressing a transgenic AID gene in the cell and expressing the antibody heavy chain gene in the cell, establishing and culturing clonal colonies of the cell, and identifying one or more clonal colonies comprising the class switch in the antibody heavy chain gene.

Applicants note that all these claims rely in part, upon the step of expressing a transgenic AID gene in a cell, and upon the Applicants' discovery that such transgenic AID expression induces somatic hypermutation in a gene of interest in that cell.

Applicants respectfully submit that the instant rejection has been improperly made using the benefit of hindsight, because *at the time that the present invention was made*, an ordinarily skilled artisan would have no expectation of success in creating the claimed methods based on the state of the art existing at that time.

i) Wabl et al (US Patent 5,885,827)

The Examiner states that "Wabl et al (US Patent No, 5,885,827) teaches a method for performing saturation mutagenesis on a target gene by exploiting the immunoglobulin hypermutation system. A target gene is cloned into an expression vector containing immunoglobulin enhancer fragments that effect hypermutation, and this construct is then transfected into an immunoglobulin mutator cell, the target gene is permitted to hypermutate, and the mutated proteins are then selected (see abstract)."

The Examiner also alleges that Wabl teaches affinity maturation, that the gene to be mutated is operatively linked to a promoter, and is within 2kb of the promoter, comprises immunoglobulin enhancer regions, teaches the use of mutator positive cell lines, the use of hybridomas, that the gene of interest can be a cytokine, and teaches the use of a tet promoter [Pages 10-11 of the Office Action].

Applicants note however, that as acknowledged by the Examiner, Wabl fails to provide any teaching, or suggestion that the use of a transgenic AID would be sufficient to effect hypermutation, [¶ 28 of the Office Action] or that specific levels of mRNA expression of the gene of interest were preferred [¶ 35 of the Office Action]. Further, Applicants respectfully remind the Examiner that a prior art reference must be considered in its entirety, i.e. as a whole, including portions that would lead away from the claimed invention. W.L. Gore & Associates, Inc. v. Garlock Inc., 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984).

By contrast, Wabl teaches that the regulation of SHM is restricted to B cells of certain types, and that within such cells, somatic hypermutation is regulated by the complicated interplay, and precise spatial orientation, of many B cell specific genetic elements. See, for example, the following sections of Wabl:

> Mutator action is restricted to a region flanked by the V gene leader intron and intron enhancer (Lebecque et al., (1990) J. Exp. Med. 172, 1717-1727; Weber et al., (1991) J. Immunol. 146, 3652-3655; Rogerson et al., (1994) Mol. Immunol. 31 85-98; Rada et al., (1994) Eur. J. Immunol. 24 1453-1457; Gonzalez-Femandez et al., (1994) Proc. Natl. Acad. Sci. 91 12614-12618). However, the recognition sequence does not seem to be contained in the 5' portion containing the promoter, and both the kappa intron and kappa 3' enhancer regions were found to be essential for full hypermutation at the K locus (Betz et al., (1994) Cell 77, 239-248; Sharpe et al., (1991) EMBO J. 10, 2139-2145). Thus, there may well be more than one recognition sequence. Somatic mutation has also been linked to the direction of DNA replication (Rogerson et al., (1991) EMBO J. 10, 4331-4341).[Column 3, lines 5- 19 of Wabl]
>
> The proximate cause for hypermutation, the putative immunoglobulin mutator system (mutator), increases the mutation rate at the gene segments encoding the endogenous V region by a factor of at least 10^5. The mutator does not work efficaciously at the Cμ gene segment (Jack et al., (1987) Proc. Natl. Acad. Sci. USA 84, 4934-4938), nor at the B2m locus (Wabl et al., (1987) Immunol. Rev. 96, 91-107). It does not seem to be active at the plasma cell stage (Wabl et al., (1985) Proc. Natl. Acad. Sci. USA 82 479-482), which represents the final stage in the differentiation of a B lymphocyte. [Column 4, lines 19-28 of Wabl]

Thus, Wabl provides no teaching or suggestion, that the expression of AID is, sufficient to induce a mutator phenotype, or any specific teaching on desired levels of mRNA expression of the gene of interest. By contrast, and as stated above, Wabl actually teaches away from this concept by teaching that SHM is regulated by the complex interplay of many B cell specific genetic elements, as well as temporally during cellular development.

Additionally, and in contrast to the Examiner's characterization, Applicants assert that Wabl does not teach that mutator cells can be fused into hybridomas.

> Most groups have found that fusion of a myeloma eliminates the hypermutation capability that is possessed by a cell line derived from pre-B lymphocytes. It has been previously reported that immunoglobulin genes are not hypermutable in hybridomas (Milstein et al., National Cancer Institute Monographs 48 321-330 [1978]; Adetugbo et al., Nature 265, 299-304 [1977]; Shulman et al., Molec. Cell. Biol. 2, 1033-1043 [1982]; Wabl et al., Proc. Natl. Acad. Sci. USA 82 479-482 [1985]). The present inventors also reported that when 18-18 was fused to the mouse plasmacytoma Ag 8.653, the resulting hybridoma showed no hypermutation (Wabl et al., Proc. Natl. Acad. Sci. USA 82 479-482 [1985]). The fusion does not interfere with the expression of the gene of interest, but it arrests the random mutagenesis. (See column 9, lines 5-19 or Wabl)

Thus, the ordinarily skilled artisan would have no expectation of success in creating the claimed methods, i.e., the utilization of a transgenic AID to drive SHM or class switch recombination in myeloma or hybridoma, either based on the teaching of Wabl alone, or in combination with any of the cited references. Applicants further note that claim 97 is amended herein to recite a myeloma, and that new dependent claim 307, relates to a hybridoma.

Finally, in making the rejection, the Examiner combines the teaching of Wabl and Muramatsu et al., [¶ 28 of the Office Action] by stating that:

> "Wabl does not teach that the altering of the antibody includes a class switch, or that the mutation factor capable of causing the hypermutation is an AID cysteine [cytidine] deaminase transgene, either constitutively expressed, or inducibly expressed while under the control of a tet promoter."

Applicants respectfully agree and further state that such teaching is also lacking in the additional references cited by the Examiner, including Muramatsu et al., as discussed below:

ii) Muramatsu et al., (Class Switch Recombination and Hypermutation require Activation-Induced Cytidine Deaminase (AID), a potential RNA Editing Enzyme. Cell 2000 102: 553-563)

The Examiner states that "Thus Muramamatsu concludes that AID cytidine deaminase is a mutation factor capable of causing class switch recombination and hypermutation, and that an AID cytidine deaminase transgene under the control of a tet promoter is capable of directly causing class switch recombination in b-cells." [¶ 33, last line & top of page 13 of the Office Action]

The Examiner also alleges that the AID transgene as constructed in the Clontech IRES-EGFP expression vector described in Muramatsu et al., is flanked by a foreign DNA of at least 2000bp. Applicants note that claim 13, is amended herein to recite that the flanking sequence is located 5′ to the AID gene, whereas the in the Clontech IRES-EGFP expression vector, the flanking sequence is 3′ to AID. [See attached vector map of the Clontech vector].

The Examiner acknowledges that neither Muramatsu et al., nor Wabl, either alone or in combination, specifically teaches specific mRNA levels of the gene or interest. The Examiner continues "Applicants have not shown that these mRNA concentrations are required for the success of the methodology of inducing mutations in gene, and applicant teaches that the

measurement of mRNA is simply to monitor transgene expression:"[Last 2 lines of ¶ 35, of the Office Action]

Applicants respectfully disagree with the Examiner's characterization. The instant application specifically discloses that transgenic AID mediated somatic hypermutation is dependent on a high rate of transcription, and provides a rationale for the importance of this in the claimed methods:

> The work reported here indicates that the SHM process is not dependent on a specific cis-acting sequences(s) to target mutation to the immunoglobulin gene, and will proceed with any cis-acting sequence that confers a high rate of transcription to the target gene. Two of our findings support this hypothesis: 1) an immunoglobulin transgene mutates in a non-B cell, and 2) the AID transgene driven by a strong promoter mutates in B and non-B cells. [Page 17, [0212], of US 2005/0095712A1]

> In addition, it must also be considered that accumulation of mutations downstream of promoters will only occur in regions that do not confer a selective disadvantage, such as regions that do not contain open reading frames or regulatory sequences for housekeeping genes. Mutations in these regions should reduce the viability of the cell, and as a consequence, the apparent rate of mutation at these loci will seem to be low or absent. . [Page 17, [0213 last 6 lines], of US 2005/0095712A1]

Thus, the Applicants clearly recognized that the expression level of the gene of interest was relevant to both the rate of somatic hypermutation, as well as to the success of the overall methodology, which includes the step of establishing and culturing clonal colonies of the cell expressing the mutant proteins of interest.

Applicants note that specific teaching with respect to the level of expression of the gene of interest was not taught in the hypermutating vectors of Wabl, because in this case, somatic hypermutation was directed to the gene of interest via the careful insertion and location of various cis acting genetic elements present in the vectors of Wabl. By contrast, the present invention is directed to somatic hypermutation, wherein SHM is mediated via the transgenic expression of AID in conjunction with expression of the gene of interest. Thus specific ranges of expression of the gene of interest, as determined by mRNA levels, are an inventive feature of certain embodiments of the present methods and inventions.

Additionally, it is well settled that the disclosure of the invention set forth by Applicants in their application must be given the presumption of correctness and operativeness by the PTO,

and the only relevant concern of the PTO under the circumstances should concern the truth of the assertions contained in the application. *See In re Bowen*, 492 F.2d 859, 181 U.S.P.Q. 48 (C.C.P.A. 1974). The Examiner fails to proffer any evidence to controvert the truth of Applicants' assertions in the instant specification.

Finally, the Examiner concludes that:

> "...One of ordinary skill in the art could have combined the elements as claimed by known methods and that in combination, each element merely would have performed the same function as it did separately, and that one of ordinary skill in the art would have yielded the predictable result of inducing somatic hypermutation in immunoglobulin antibodies and identifying clones producing new monoclonal antibodies (see KSR International Co. v. Teleflex Inc., 82 USPQ2d 1385 (U.S. 2007)). Given the teachings of the prior art and the level of skill of the ordinary skilled artisan at the time the instant invention was made, it must be considered that said ordinary skilled artisan would have had a reasonable expectation of success in practicing the claimed invention." [¶ 39 of the Office Action]

Applicants respectfully disagree, and provide below specific evidence to demonstrate that at the time the present application was filed, the art provided no expectation of success to combine the references as cited by the Examiner, and that by contrast, the state of the art, at the time the instant invention was made, clearly taught away from the claimed inventions.

Muramatsu et al., characterized the phenotype of an AID knock out mouse, and described the effect of the induced over expression of AID in a B lymphocyte cell line <u>on the rate of class switch recombination in that cell</u>. Applicants note that class switch recombination, and somatic hypermutation are completely different and distinct processes;

CSR involves the replacement of the Ig heavy chain constant region (CH) gene to be expressed from Cm to other CH genes, resulting in switch of the Ig isotype from IgM to either IgG, IgE, or IgA.

Somatic hypermutation involves the accumulation of point mutations in the V exon and gives rise to high-affinity antibodies for a given antigen in a process called affinity maturation. (See Muramatsu et el., second column, page 553)

Applicants would like to respectfully emphasize that Muramatsu et el., ***neither directly demonstrated, nor proposed that the expression of a transgenic AID in a cell was sufficient to induce somatic hypermutation of a gene of interest in that cell.***

In fact, Muramatsu et al.'s results and conclusions with respect to the potential role of AID in somatic hypermutation were limited to speculation as to the possible involvement of AID

in any one, of many potential points of involvement in the overall process of affinity maturation – ***and based solely*** on data derived from experiments conducted in AID knock-out mice.

Furthermore, prior to the Applicant's discovery of the direct role of AID in DNA deamination, the close homology of AID to the Apobec-1 family of RNA editing enzymes suggested that AID acted at the level of an RNA editing enzyme, by analogy with the role of Apobec-1 in apo-B processing. See for example, Muramatsu et al.

> The most straightforward possibility for the function of AID is, therefore, an RNA editing enzyme with a substrate specificity determined **by an additional co-factor like ACF for APOBEC-1** (Mehta et al., 2000). In that case, mRNA encoding an unknown protein might be converted to that for CSR recombinase and hypermutator by the function of AID **and its cofactor**. [Muramatsu et al. page 561, first column, lines 10-17, Emphasis added)

Thus, Muramatsu et al. proposed that AID acts as an RNA editing enzyme, and therefore is not the actual agent responsible for somatic hypermutation or class switch recombination respectively. Applicants further note that AID induced mRNA editing of a target gene would not lead to productive, stable, inherited changes at the DNA level in a gene product of interest to enable its directed evolution to create improved forms of that gene of interest, as currently claimed.

In fact, a review of the state of the art prior to Applicants' discovery of AID mediated hypermutation, reveals no clear teaching to motivate a person of skill in the art to create the claimed invention, or to provide a reasonable expectation of success.

By contrast, the teaching of the art at the time the present specification was filed, and specifically the teaching by the same senior author of Muramatsu et al., (Tasuku Honjo), would not have provided an expectation of success to modify or adapt the closest prior art to arrive at something falling within the scope of the claims.

To support this conclusion, Applicants further submit the following papers; which were published after Muramatsu et al., and by, or with, Honjo's group, and further define the state of the art at the time that the present inventions were made.

a. Kinoshita and Honjo (2001) Linking Class Switch recombination with somatic hypermutation Nature Reviews **2** 493-503.

This review by Kinoshita and Honjo summarizes the state of the art view on class switch recombination and somatic hypermutation in light of the results of Muramatsu et al. The review clearly establishes that the thinking at the time of the present application was focused on the concept that AID acted at the level of RNA editing, in conjunction with one or more additional factors, to create an endonuclease capable of creating DNA strand breaks. Kinoshita and Honjo proposed that these DNA breaks could then subsequently lead to class switch recombination, or be repaired in an error prone fashion, to induce somatic hypermutations. See specifically, for example, the following sections of Kinoshita and Honjo:

> The predicted amino acid sequence (198 residues) of AID has homology to that of APOBEC-1, which is a catalytic subunit of the RNA-editing complex for the apolipoprotein B messenger RNA precursor – APOBEC-1 converts the specific cytosine at position 6666 of apolipoprotein B mRNA to uracil by its intrinsic cytidine deaminase activity, giving rise to mRNA that encodes the CHYLOMICRON component. Although APOBEC-1 cannot bind a specific sequence in the target mRNA, this specificity is conferred by a protein with which it associates, APOBEC-1 COMPLEMENTATION FACTOR (ACF). Like APOBEC-1, AID synthesized in vitro has cytidine deaminase activity. Moreover, both the APOBEC-1 and AID genes are closely mapped to human chromosome 12p13. **These structural, genetic and functional relationships of AID and APOBEC-1 indicate that AID might be an RNA-editing enzyme. AID in a complex with an ACF-like protein, might edit a certain mRNA, giving rise to a new mRNA that encodes the class switch recombinase.** [Kinoshita and Honjo, page 499, 2nd column, lines 26- 45, emphasis added]
>
> On the basis of this speculation, we propose a model in which the endonuclease(s) encoded by AID-edited mRNA has recognition specificity to the stem-loop structure, and cleaves in its proximity in the V and S regions during somatic hypermutation and class-switch recombination reactions, respectively [Kinoshita and Honjo, page 500, first column, lines 4-10]
>
> Conversely, in the case of somatic hypermutation, the V gene might be attacked by the same or similar endonucleases, but less efficiently, probably owing to less extensive secondary structure (FIG, 5b). **This results in the generation of single-stranded nicks, which could be repaired by low fidelity DNA polymerases exonucleases and ligase. Mutations are most likely to be introduced during this repair.** [Kinoshita and Honjo, page 501, first column, lines 4-11, emphasis added]

Thus, the teaching of the Honjo group does not teach that AID is sufficient to induce a mutator phenotype in a cell, by contrast, Kinoshita and Honjo, and Muramatsu et al., both teach that AID acts within the context of at least three additional un-identified B-cell specific factors, (1) an accessory DNA recognition co-factor, (2) an endonuclease capable of being edited by AID, (3) and a set of low fidelity DNA polymerases and exonucleases.

Applicants further note that AID induced mRNA editing of a target gene of interest, as mentioned previously would not lead to productive, stable, inherited changes at the DNA level in that gene product, to enable its directed evolution to create improved forms of that gene, as currently claimed.

b. Papavasiliou and Schatz (2002) The Activation-induced Deaminase Functions in a Postcleavage Step of the Somatic Hypermutation Process J. Exp. Med. 195 (9): 1193-1198.

Papavasiliou and Schatz demonstrate that cells **lacking AID**, or expressing a dominant negative form of the protein, are still able to incur DNA lesions in SHM target sequences (see Abstract). Applicants note that Papavasiliou and Shatz collaborated with Honjo et al. in performing this work.

Papavasiliou and Schatz also teach that in the prior art that existed immediately around the date of the present invention, the role, if any, of AID in directly mediating somatic hypermutation was completely unclear. Furthermore, Papavasiliou and Schatz explicitly teach that AID acts down stream of DNA mutagenesis, and via an indirect effect that is mediated by mRNA editing of a repair factor. As reproduced below from Papavasiliou and Schatz:

> **Yet, the function of AID in somatic hypermutation is far from clear, and its effect might not even be direct, as AID is thought to be an RNA-editing enzyme** (12). It has been postulated that AID edits the mRNA of the endonuclease responsible for the DNA lesion in both CSR and SHM (10). Alternatively, AID might be responsible in editing the mRNA of a factor (or factors) responsible for orchestrating DSB repair. **Here we report that SHM-associated DSBs are still present in cells lacking AID, or expressing a dominant negative form of the protein. Our results indicate that AID acts down stream of the initial DNA lesions in SHM, possibly by editing the RNA of a repair factor.** (Papavasiliou and Schatz, last sentence of page 1193, first 12 lines of page 1194, first column; Emphasis added).

Thus, Papavasiliou and Schatz teach that AID is not directly involved in mediating somatic hypermutations, but is instead involved in the activation of an error prone repair factor, via RNA editing, a process that would not lead to the productive mutation of proteins in cells lacking the primary mutagenic protein and other SHM specific accessory factors.

Thus, the combined teaching of Muramatsu et al., Kinoshita and Honjo and Papavasiliou and Shatz, in combination with the art as a whole, at the time the present invention was made, fails to make the claimed invention obvious, because AID induced mRNA editing of a target gene, would not lead to productive, stable, inherited changes at the DNA level in a gene product, to enable its iterative directed evolution to create improved forms.

Applicants thus respectfully submit that the instant rejection has been improperly made using the benefit of hindsight because, *at the time that the present invention was made*, the use of transgenic AID to create the claimed methods for mutagenesis was both surprising and unexpected, and thus an ordinarily skilled artisan would have no expectation of success in creating the claimed methods based on the state of the art existing at that time.

Furthermore, the subsequently published work of Tasuku Honjo (the senior author of Muramatsu et al.) clearly teaches away from the concept that the use of a transgenic AID gene is sufficient to enable the development of a practical or useful method of inducing inheritable mutations in a gene of interest (i.e. the method (i) above); or an antibody gene (method ii, above), or could be used as a system for changing the affinity or specificity of an antibody (method iii above), or that a transgenic AID gene is sufficient to induce a class switch in an antibody gene in a myeloma (iv, above).

Finally, none of the additional references cited by the Examiner, including those of Wabl, Wang et al., Griffiths et al., or Honjo et al., either alone or in combination, teach use of a transgenic AID gene to mediate DNA mutagenesis. By contrast, these additional references merely teach additional individual components of the present invention, as described in the dependent claims. Thus, the Examiner has failed to establish a prima facie case of obviousness.

Accordingly, Applicants respectfully requests that the rejection of claims 1-4, 6-9, 13, 15, 19-22, 24-25, 58, 97, 125, 262-272, and 276-284, 287-288, 289-291, and 293-300 under 35 U.S.C. § 103(a) be reconsidered and withdrawn. Applicants further submit that the rejection is not applicable to new claims 301-307.

B. Claims 5 and 23 are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Wabl et al., (US patent No. 5,885,827) in view of Muramatsu et al., (Class Switch Recombination and Hypermutation require Activation-Induced Cytidine Deaminase (AID) a Potential RNA Editing Enzyme, Cell, 2000 102 553-563), further in view of Wang et al (US Patent Publication 2003/0119190).

The Examiner states that "Neither Wabl nor Muramamatsu teaches that the promoter is an immunoglobulin promoter (claim 5), nor that the method is performed in a human cell (claim 23). [Last two lines of ¶ 41 of the office action].

The Examiner also states that "Thus, Wang et al., does not teach the use of AID transgene prior to the priority date of the instant application, and is NOT being used to supply this limitation to the rejection" [¶ 44, of the Office Action].

The teachings of Wabl and Muramatsu et al. are discussed in detail above, and as stated previously, neither reference, either alone, or in combination, provides any expectation of success of one of skill in the art, to create the claimed methods.

Additionally as acknowledged by the Examiner, Wang et al., does not teach the use of a transgenic AID in the claimed methods, and thus is unable to overcome the deficiencies of Wabl and Muramatsu et al., and thus is not sufficient to render claims 5 or 23 obvious in combination with these references.

Accordingly, Applicants respectfully requests that the rejection of claims 5 and 23 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

C. Claims 273, 274 and 275 are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Wabl et al., (US patent No. 5,885,827) in view of Muramatsu et al., (Class Switch Recombination and Hypermutation require Activation-Induced Cytidine Deaminase (AID) a Potential RNA Editing Enzyme, Cell, 2000 102 553-563), as applied to claim 58, and further in view of Griffiths (US Patent 5,885,827).

The Examiner states that "Griffiths teaches his technique allow for rapid isolation of binding specificities of different types of antibodies, including single chain, multivalent and catalytic antibodies (column 13, lines 3040, column 15, lines 35-58)." [¶ 49, of the of the Office Action].

The teachings of Wabl and Muramatsu et al. are discussed in detail above, and as stated previously, neither reference, either alone, or in combination, provides any expectation of success of one of skill in the art, to create the claimed methods.

Applicants note, that because Griffith does not teach the use of a transgenic AID in the claimed methods, it is thus unable to overcome the deficiencies of Wabl, Muramatsu et al., and / or Wang et al., and thus is not sufficient to render claims 273, 274 and 275 obvious in combination with these references.

Accordingly, Applicants respectfully requests that the rejection of claims 273, 274 and 275 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

D. Claims 18, 285 and 286 are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Wabl et al., (US patent No. 5,885,827) in view of Muramatsu et al., (Class Switch Recombination and Hypermutation require Activation-Induced Cytidine Deaminase (AID) a Potential RNA Editing Enzyme, Cell, 2000 102 553-563), as applied to claim 1, and further in view of Honjo (US Patent 6,815,914).

The Examiner states that "Honjo et al (US Patent 6,815,914) teaches methods of expressing AID transgenic cDNAs in eukaryotic cells including host cells of yeast, bacterial and insect cells, murine and human cells, including myeloma and hela cells (see abstract and column 19, lines 9-57; column 20 lines 29-47). Honjo further teaches that these host cells are common in the art for producing recombinant proteins (Column 30 line 50 – column 31 line 5; and Column 31 lines 40-55) [¶ 53, of the Office Action].

The teachings of Wabl and Muramatsu et al. are discussed in detail above, and as stated previously, neither reference, either alone, or in combination, provides any expectation of success to one of skill in the art, to create the claimed methods.

Applicants note that teaching of Honjo are limited to simple methods of expressing and preparing recombinant protein, and that there is no suggestion, or teaching in Honjo for the use of a transgenic AID in the Applicants claimed methods. Thus Honjo is unable to overcome the deficiencies of Wabl, Muramatsu et al., Wang et al., and / or Griffith, it is thus is not sufficient to render claims 273, 274 and 275 obvious in combination with these references.

Accordingly, Applicants respectfully requests that the rejection of claims 18, 285 and 286 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

Based upon the foregoing arguments, it is the Applicants' position that at the time that the present invention was made, the state of the art as evidenced, for example, by the published work of Tasuku Honjo (the senior author of Muramatsu et al.) clearly teaches away from the concept that the use of a transgenic AID gene is sufficient to enable the development of a practical or useful method of inducing inheritable mutations in a gene of interest (i.e. independent claim 1); or an antibody gene (independent claim 58), or could be used as a system for changing the affinity or specificity of an antibody (independent claim 125), or that a transgenic AID gene is sufficient to induce a class switch in an antibody gene in myeloma (independent claim 97).

Finally, none of the additional references cited by the Examiner, including those of Wabl, Wang et al., Griffiths et al., or Honjo et al., either alone or in combination, teach use of a transgenic AID gene to mediate DNA mutagenesis of a gene of interest. By contrast, these additional references merely teach additional individual components of the present invention, as described in the dependent claims.

Thus, an ordinarily skilled artisan would have no expectation of success in creating the claimed methods based on the state of the art existing at that time. Accordingly, Applicants request that the rejection of pending under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

CONCLUSIONS

In view of the above remarks, reconsideration and allowance of the application are respectfully requested. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at 212-336-8070.

The Commissioner is authorized to charge any additional fees that may be required in connection with this submission, including petition fees and extension of time fees, or to credit any overpayments to Deposit Account No. 01-1785.

Respectfully submitted,

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Dated: New York, New York
February 12, 2008

By: _____
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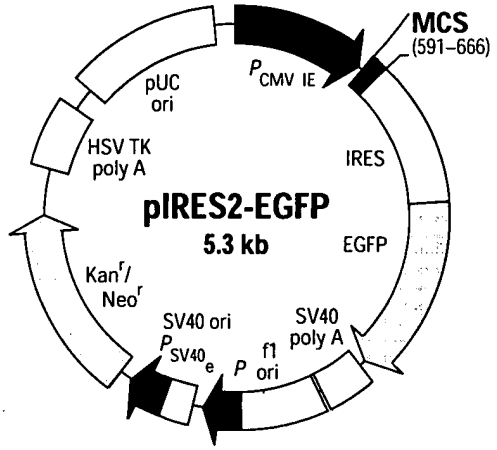

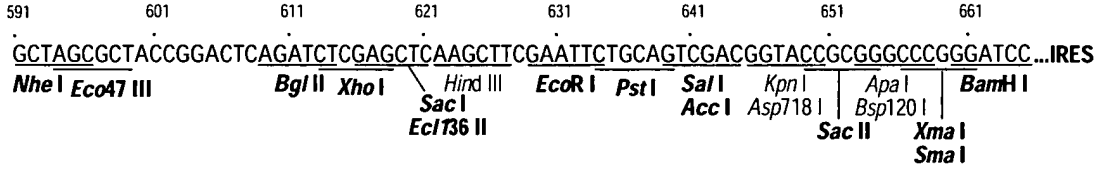

Restriction Map and Multiple Cloning Site (MCS) of pIRES2-EGFP Vector. Unique restriction sites are in bold. Note that the *Eco*47 III site has not been confirmed in the final construct.

Description:

pIRES2-EGFP contains the internal ribosome entry site (IRES; 1, 2) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (EGFP) coding region. This permits both the gene of interest (cloned into the MCS) and the EGFP gene to be translated from a single bicistronic mRNA. pIRES2-EGFP is designed for the efficient selection (by flow cytometry or other methods) of transiently transfected mammalian cells expressing EGFP and the protein of interest. This vector can also be used to express EGFP alone or to obtain stably transfected cell lines without time-consuming drug and clonal selection.

EGFP is a red-shifted variant of wild-type GFP (3–5) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) EGFP encodes the GFPmut1 variant (6) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (7). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (8) to further increase the translation efficiency in eukaryotic cells. The MCS in pIRES2-EGFP is between the immediate early promoter of cytomegalovirus ($P_{CMV\ IE}$) and the IRES sequence. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the bicistronic mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli*. The pIRES2-EGFP backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production. pIRES2-EGFP replaces (but is not derived from) the pIRES-EGFP Vector previously sold by BD Biosciences Clontech. pIRES2-EGFP is functionally similarly to pIRES-EGFP; however, pIRES2-EGFP gives brighter EGFP fluorescence than the older vector. Note that the *Xba* I site at position

(PR29951; published 03 October 2002)

1987 is methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vector with this enzyme, you will need to transform the vector into a *dam*⁻ host and make fresh DNA.

Use:
Genes inserted into the MCS should include the initiating ATG codon. pIRES2-EGFP and its derivatives can be introduced into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418 (9).

Location of features:
- Human cytomegalovirus (CMV) immediate early promoter: 1–589
  Enhancer region: 59–465; TATA box: 554–560; Transcription start point: 583
  C→G mutation to remove *Sac* I site: 569
- MCS: 591–665
- IRES sequence: 666–1250
- Enhanced green fluorescent protein (EGFP) gene
  Kozak consensus translation initiation site: 1247–1257
  Start codon (ATG): 1254–1256; Stop codon: 1971–1973
  Insertion of Val at position 2: 1257–1259
  GFPmut1 chromophore mutations (Phe-64 to Leu; Ser-65 to Thr): 1446–1451
  His-231 to Leu mutation (A→T): 1948
- SV40 early mRNA polyadenylation signal
  Polyadenylation signals: 2127–2132 & 2156–2161; mRNA 3' ends: 2165 & 2177
- f1 single-strand DNA origin: 2224–2679 (Packages the noncoding strand of EGFP.)
- Bacterial promoter for expression of Kan^r gene:
  –35 region: 2741–2746; –10 region: 2764–2769
  Transcription start point: 2776
- SV40 origin of replication: 3020–3155
- SV40 early promoter/enhancer
  72-bp tandem repeats: 2853–2996; 21-bp repeats (3): 3000–3063
  Early promoter element: 3076–3082
- Kanamycin/neomycin resistance gene: 3204–3998
  G→A mutation to remove *Pst* I site: 3386; C→A (Arg to Ser) mutation to remove *Bss*H II site: 3732
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signals: 4234–4252
- pUC plasmid replication origin: 4583–5226

Propagation in *E. coli*
- Suitable host strains: DH5α, HB101, and other general purpose strains. Single-stranded DNA production requires a host containing an F plasmid such as JM101 or XL1-Blue.
- Selectable marker: plasmid confers resistance to kanamycin (30 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: pUC
- Copy number: ~500
- Plasmid incompatibility group: pMB1/ColE1

References:
1. Jackson, R. J., *et al.* (1990) *Trends Biochem. Sci.* **15**:477–483.
2. Jang, S. K., *et al.* (1990) *J. Virol.* **62**:2636–2643.
3. Cormack, B., *et al.* (1996) *Gene* **173**:33–38.
4. Yang, T. T., *et al.* (1996) *Nucleic Acids Res.* **24**:4592–4593.
5. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
6. Jackson, R. J., *et al.* (1990) *Trends Biochem.* **15**:477–483.
7. Jang, S. K., *et al.* (1988) *J. Virol.* **62**:2636–2643.
8. Huang, M. T. F. & Gorman, C. M. (1990) *Nucleic Acids Res.* **18**(4):937–947.
9. Gorman, C. (1985). In *DNA cloning: A practical approach, vol. II.* Ed. D.M. Glover. (IRL Press, Oxford, U.K.) pp. 143–190.

Note: The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with partial sequences obtained by BD Biosciences Clontech. This vector has not been completely sequenced.

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LINKING CLASS-SWITCH RECOMBINATION WITH SOMATIC HYPERMUTATION

Kazuo Kinoshita and Tasuku Honjo

The recent discovery of a molecular link between two apparently different genetic alteration events — class-switch recombination and somatic hypermutation — has led to the idea that the recognition and cleavage of target DNA in these two events might be mediated by similar or identical molecules to those involved in RNA editing. This could mean that the complexity of mammalian genetic information may be enriched by an interplay between RNA editing and DNA modification.

ENHANCER
A regulatory element of DNA that activates transcription from distant promoters, independent of position and orientation.

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Recent analyses of the human genome sequence have revealed, surprisingly, far fewer genes than previously speculated[1,2]. This result indicates that regulation of our genetic information might be far more complex than anticipated, allowing it to carry out sophisticated functions in, for example, the central nervous and immune systems. The most drastic strategy to regulate our genetic information is its alteration at the level of the somatic cell — this can be done at the DNA level by mutations and various types of recombination, and genetic information can also be altered at the level of transcripts. Indeed, RNA can be edited to generate diverse transcripts from a single gene in many organisms including protozoa, plants and mammals[3] (BOX 1, TABLE 1).

The immune system takes advantage of genetic alterations to amplify its diversity. There are two stages of lymphocyte differentiation, each of which uses distinct mechanisms for genetic alteration. During the early stages of T- and B-lymphocyte differentiation, *V(D)J* recombination takes place to assemble variable (*V*), diversity (*D*) and joining (*J*) segments of the *V* exon of the T-cell receptor and immunoglobulin genes, respectively, giving rise to diverse repertoires of lymphocytes, each expressing receptors for specific antigens (FIG. 1). *V(D)J* recombination is tightly regulated during lymphocyte differentiation by transcriptional regulation of the recombinase genes that encode the RAG-1 and RAG-2 proteins. These proteins recognize a defined primary sequence that is composed of a nonamer and a heptamer with a 12- or 23-base-pair spacer[4]. Upon encounter with antigens, mature B lymphocytes are activated, and they begin to proliferate extensively in a special microenvironment called the germinal centre (BOX 2). During the proliferation of B lymphocytes, the immunoglobulin gene undergoes two types of DNA modification, namely class-switch recombination and somatic hypermutation.

Class-switch recombination alters the immunoglobulin heavy-chain (H) constant (*C*) region gene that will be expressed from the $C\mu$ region to one of the other C_H genes. This results in a 'switch' of the immunoglobulin (Ig) isotype from IgM/IgD to IgG, IgE or IgA, without changing the antigen specificity. Each isotype determines how captured antigens are eliminated or the location to which the immunoglobulin is delivered. Somatic hypermutation, on the other hand, accumulates massive point mutations in the *V* genes of the H and light (L) chains, giving rise to high-affinity antibodies against a given antigen in a process called affinity maturation, in which B cells expressing high-affinity immunoglobulins on their surface are selected by limited amounts of the antigen. Mutations are introduced at a high frequency in a defined region between the immunoglobulin promoter and the intronic immunoglobulin ENHANCER.

Table 1 | **RNA editing in mammalian cells**

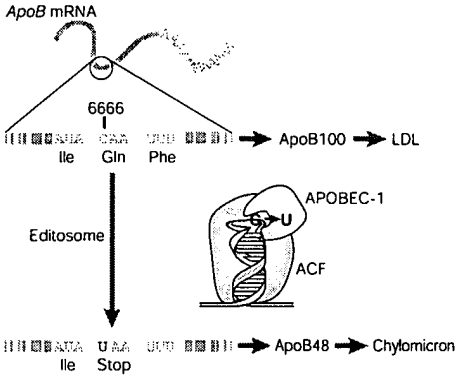
Enzyme	Base modification	Tissue	Target pre-mRNA	Outcome of editing	Reference
APOBEC-1	C to U	Small intestine (and liver in mouse)	ApoB100	Generation of stop codon to produce *ApoB48* mRNA	97
ADAR2	A to I	Brain	Ionotropic glutamate receptors	Amino-acid substitution (Arg to Gln) to decrease Ca^{2+} permeability	98
ADAR2	A to I	Brain	ADAR2	Creation of splice acceptor	99
ADAR1	A to I	Fetal liver	Unknown	Support of embryonic erythropoiesis	96
Unknown	A to I	Brain	5-HT_2C receptor	Downmodulate signalling activity to G protein	100
Unknown	A to I	Liver	Sialyltransferase	Amino-acid substitution (Tyr to Cys)	101
Unknown	A to I	Liver	Hepatitis D virus, p24	Conversion of stop to tryptophan codon to produce p27, a protein required for packaging of viral genome	102
Unknown	C to U G to A	T cell	HIV proteins	Unknown	103
Unknown	Insertion of A	?	Ebola virus, GP	Production of full-length GP protein by frameshifting	104
Unknown	U to C	Kidney	WT1	Amino-acid substitution (Leu to Pro)	105
AID	C to U?	B cell	?	CSR and SHM	5

Because class-switch recombination and somatic hypermutation deal with diversification of the C_H and V genes, respectively, these two genetic alterations have been considered to be mechanistically distinct. However, the recent discovery[5] of activation-induced cytidine deaminase (AID), a potential RNA-editing enzyme, and subsequent studies of its function have revealed an unexpected link — not only between class-switch recombination and somatic hypermutation, but also between DNA modification and, probably, RNA editing. This review summarizes recent studies on the molecular mechanism of class-switch recombination using artificial class-switching substrates and functional analysis of AID. The molecular mechanism of somatic hypermutation is also reviewed and compared to that of class-switch recombination. Finally, we speculate on how these two DNA-alteration systems are linked by AID.

Box 1 | **RNA editing in mammals**

RNA editing — the post-transcriptional modification of RNA sequences — is a widespread phenomenon in eukaryotes from protozoa, plants and flies to mammals. RNA editing was first reported in the mitochondria of *Trypanosoma*, in which multiple insertion of uridine to a given RNA creates new reading frames and amino-acid insertions. Another type of RNA editing in plant mitochondria and mammals replaces one specific base with another by deamination of a base (cytidine to uridine, or adenosine to inosine) or by unknown mechanisms. This leads either to amino-acid replacement or to the creation of new initiation or termination codons, or splicing sites.

In mammalian cells, the number of candidates involved in RNA editing is increasing. The best-characterized RNA-editing enzyme is APOBEC-1 which, together with its associating protein APOBEC-1 complementation factor (ACF) and other proteins that form an editing complex called the editosome, can recognize target sequences on mRNA coding for ApoB100, a component of low-density lipoprotein (LDL). By changing cytosine at position 6666 to uracil, this APOBEC-1 converts the CAA to UAA termination codon, generating a truncated form, ApoB48 mRNA, which encodes an essential component of chylomicron. In *APOBEC-1*-deficient mice, chylomicron is not produced and hyperlipidaemia occurs.



In the nervous system, double-stranded RNA-binding adenosine deaminase, Adar2, is responsible for editing mRNA encoding ionotropic glutamate receptors. In the absence of this enzyme, mice die from epilepsy within a few weeks after birth. Adar2 edits its own pre-mRNA and changes the pattern of alternative splicing, probably regulating the amount of protein post-transcriptionally. Adar1, a homologue of Adar2, was recently shown[96] to be required for embryogenesis, and the cause of death seems to be impaired development of red blood cells. However, the molecular mechanisms of other forms of RNA editing remain largely unknown.

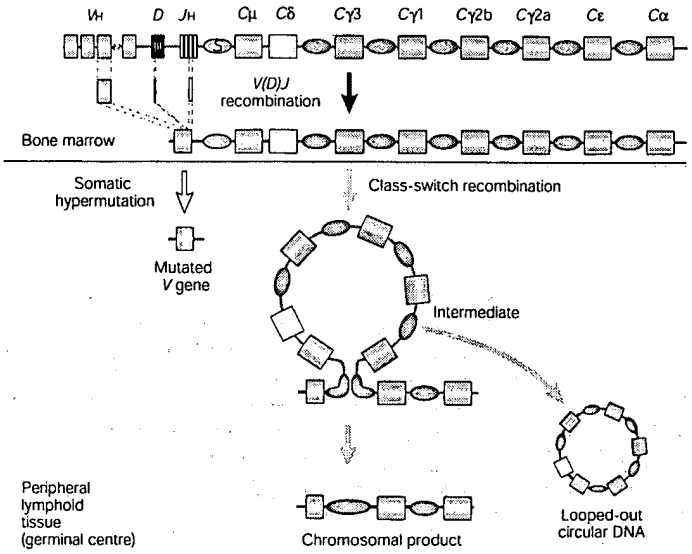


Figure 1 | **Immunoglobulin heavy-chain gene organization and its DNA alteration.** The mouse immunoglobulin heavy-chain locus is shown. Rectangles and ovals represent exons and switch (*S*) regions, respectively. *V(D)J* recombination occurs in the bone marrow, whereas somatic hypermutation and class-switch recombination occur in the peripheral lymphoid tissues. *V(D)J* recombination selects one segment for each of the *V*, *D* and *J* segments from respective pools of gene fragments and combines them into a single variable (*V*)-region exon. Somatic hypermutation introduces frequent mutations in the rearranged *V* exon, providing a pool of B cells with diverged antigen specificity from which high-affinity immunoglobulin producers are selected. Class-switch recombination brings the downstream constant (*C*) region exon in the proximity of the *V* exon by deletion between Sμ and another *S* region upstream of the target *C* region. Deleted DNA is released as a circular DNA.

S REGION
A functional region of DNA where class-switch recombination occurs, comprising repetitive sequences of palindrome-rich motifs.

CD40
A cell-surface receptor that belongs to the tumour necrosis factor receptor superfamily and is expressed on B lymphocytes, dendritic cells, activated macrophages, monocytes and endothelial cells.

CD40L
CD40L (or CD154) is a ligand of CD40. It belongs to the tumour necrosis factor superfamily and is expressed on activated T lymphocytes, monocytes and natural killer cells.

TRANSFORMING GROWTH FACTOR β (TGF-β)
A cytokine that has pleiotropic functions in the cell. In class-switch recombination, it activates transcription from the *Iα* promoter, promoting IgA class switching.

Events in class-switch recombination

The immunoglobulin *C*H locus consists of an ordered array of *C*H genes, each flanked at its 5′ region by a switch (*S*) region composed of tandemly repetitive unit sequences with many palindromes[6–15]. Class-switch recombination takes place between two *s* REGIONS, resulting in looped-out deletion of intervening DNA segments[16–18]. The Cμ gene is located at the *V*H proximal end of the *C*H gene cluster (FIG. 1), so class-switch recombination between Sμ and another *S* region that lies 5′ to a *C*H gene brings that particular *C*H gene adjacent to the *V*H exon. Because neither consensus nor homologous sequence is generally found around junctions, class-switch recombination is categorically different from homologous and site-specific recombinations such as *V(D)J* recombination, and may be classified as region-specific recombination (TABLE 2).

The class-switch recombination reaction can be divided into three steps: choice of a downstream *S* region as a target to pair with Sμ; recognition and cleavage of the target DNA by the class-switch recombinase; and repair and ligation of the broken DNA ends in a different pair, resulting in looped-out deletion. Initiation of class-switch recombination requires activation of B lymphocytes by antigens and cytokines secreted by activated T cells and/or macrophages (BOX 2).

With most protein antigens, T-cell help is required to activate B cells. Direct contact between B and T cells takes place through an interaction between CD40 and its ligand CD40L. Signal transduction through surface IgM, CD40 and cytokine receptors on B cells induces the two important events that are required for class-switch recombination: first, selection of a target *S* region[19,20]; and second, induction or activation of the class-switch recombinase[5,21]. The repair step of class-switch recombination is mediated by the non-homologous end-joining (NHEJ) repair system, which is constitutively expressed in almost all cells[22–24].

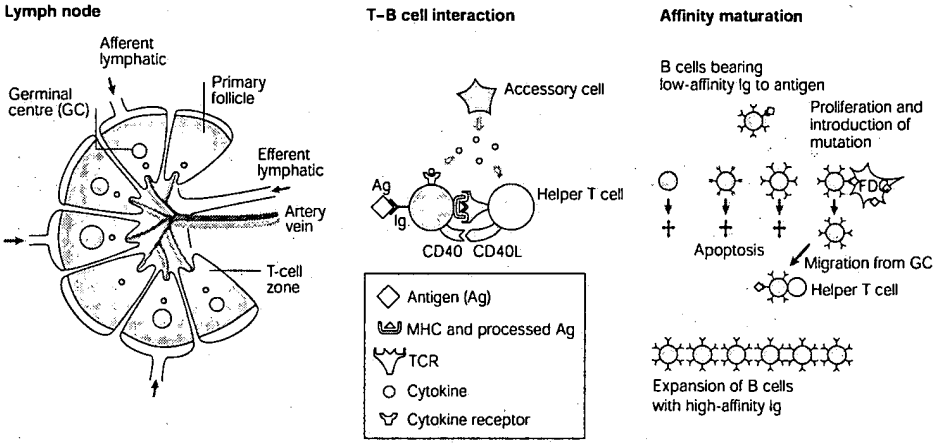
Selection of the target

Stimulation by a specific cytokine determines the target *S* region of class-switch recombination and, thus, the immunoglobulin class generated by this process. In mature B cells, the Sμ region is constitutively transcribed from the intronic (*I*) promoter located 5′ to the Sμ region, whereas transcription of downstream *S* regions is induced only after stimulation with specific cytokines such as interleukin 4 (IL-4). Each cytokine signalling event activates specific intronic promoters that are located 5′ to *S* regions, giving rise to so-called germline transcripts that contain the *I* and *C*H exons. Splicing removes the *S* region sequence from primary transcripts (FIG. 2). A close association between isotype specificity of germline transcription and the recombination target of *S* regions by stimulation with a given cytokine has led to the accessibility model, whereby germline transcription opens the chromatin structure of a specific *S* region and renders it accessible to a putative recombinase[19,20] (FIG. 2). Several gene-targeting experiments abolishing germline transcription[25–28] confirmed that this process is required during class-switch recombination. Transcription-dependent locus opening had been also shown to be required for *V(D)J* recombination[29].

Recognition and cleavage of target DNA

Recognition of the S region. To understand the *cis* elements that are required for class-switch recombination, various *in vitro* assay systems were developed using artificial DNA constructs and B cells or B-cell lines[30–36]. One of the most effective was a system using an efficiently switching mouse lymphoma cell line (CH12F3-2), in which 80% of the cells switch from expressing IgM to (almost exclusively) IgA in one week after stimulation with CD40L, IL-4 and TRANSFORMING GROWTH FACTOR β (TGF-β)[37]. DNA constructs introduced into CH12F3-2 cells contain the Sμ and Sα regions, and surface-expression markers to monitor class-switch recombination (FIG. 3a,b). Although the *S* regions are constitutively transcribed by separate promoters, class-switch recombination requires cytokine stimulation, indicating that the stimulation induces activation of new genes involved in class-switch recombination such as *AID*[34]. In fact, class-switch recombination in the endogenous immunoglobulin locus of CH12F3-2 cells is blocked by a protein-synthesis inhibitor, cycloheximide[21]. Studies using a construct without the *S* region[34], or using several other

Box 2 | **The germinal centre**



The germinal centre is a site of active proliferation and selection of B cells in peripheral lymphoid tissues such as the spleen, lymph node, tonsil and PEYER'S PATCH of the intestine. The structure of the lymph node is shown in the figure. Lymph nodes are encapsulated by fibrous tissue and radial trabeculae support various cellular components. The cortical area mainly contains B cells, and is often called a primary follicle, whereas the inner paracortical area contains T cells. When B cells in the primary follicle are activated by invading antigens and helper T cells, they begin to proliferate rapidly to form secondary follicles or germinal centres. T and B cells interact and activate each other through the binding of T-cell receptors (TCR) and processed antigens on major histocompatibility complex (MHC) class II on B cells, and through CD40 ligand–receptor interactions. Activated T cells and accessory cells secrete various cytokines to modulate the B-cell response.

An activation signal in B cells induces the transcription of target *S* regions (germline transcription), as well as the expression of activation-induced cytidine deaminase (*AID*), which is essential for class-switch recombination and somatic hypermutation. Such T–B-cell interactions occur in the germinal centre and in the vicinity of the arteriole in the T-cell area. In germinal centres, B cells undergo somatic hypermutation, which introduces massive mutations in rearranged *V* genes, and class-switch recombination. B cells expressing immunoglobulin with a high affinity to given antigens are selected by follicular dendritic cells (FDC), which hold non-processed antigens in a form of the immune complex on the surface. B cells that have lost functional immunoglobulin, or acquired only low-affinity immunoglobulin after somatic hypermutation, die by apoptosis.

types of artificial construct in combinations of B-cell lines and spleen cells[31–33,38], have clearly shown that the *S* region is essential for class-switch recombination. Furthermore, knockout mice of the core *Sμ* region showed decreased levels of class switching to all isotypes[39]. (Residual switching observed in these mice might be due to the presence of scattered *Sμ* repeat motifs outside the core *Sμ* region.)

Recognition of secondary structure. The class-switch recombinase might recognize the SECONDARY STRUCTURE of *S*-region DNAs rather than the primary sequence. Evidence for this idea includes the fact that the *S* regions of different isotypes (*Sγ*1 and *Sα*) and species (chicken and frog), as well as an inverted *Sα* region, are functional in CH12F3-2 cells, which switch almost exclusively to IgA[34,40]. Scrutiny of recombination junctions in mammalian and amphibian class-switch recombination — in both endogenous loci[41] and artificial substrates[40] — revealed that their distribution is biased to the proximity of the stem–loop structure in single-stranded *S* sequences, which is predicted by a computer program developed by Zuker, based on thermodynamic parameters by SantaLucia[42]. Because *S* sequences, irrespective of the isotypes and species, commonly contain many short stretches of inverted repeats, the stem–loop structure may be formed when they are single stranded (FIG. 3c). Furthermore, a completely artificial sequence that contains multiple cloning sites was shown to functionally replace the *S* sequence in class-switch recombination substrates[40] (FIG. 3c). The only structural feature shared by this and natural *S* sequences is the presence of several inverted repeats or the palindromes, further supporting the idea discussed above.

Staggered cleavage. Comparative analysis of two reciprocal junction sequences in class-switch recombination, one of which is lost by looping-out circular DNA, was made possible using artificial substrates that direct inversion-type recombination[43] (FIG. 4a). Analysis of these junctions revealed deletion and duplication with variable lengths exclusively at junction sites (X.C. Chen, K.K. and T.H., unpublished data) (FIG. 4b). The introduction of duplication during class-switch recombination indicates that DNA breaks in *S* regions are due mainly to STAGGERED NICKS on two strands of DNA.

PEYER'S PATCH
A small collection of lymphoid cells that appear in the submucosal tissue (lamina propria) of the small intestine, constituting the secondary lymphoid organ of the gut.

SECONDARY STRUCTURE
Atypical structure of DNA formed by intra-strand base-pairing, such as stem–loops.

Table 2 | **Comparison between CSR, SHM and *V(D)J* recombination**

	CSR	SHM	*V(D)J* recombination
Target	*S* region and its flanks	*V* exon and its flanking intron	*V,D,J* segments
Transcription	Required	Required	Not required?
AID	Required	Required	Dispensable
Association of DNA cleavage with mutations	Yes	Yes	Yes
Specificity of primary sequence	Absent	Absent	Defined nanomer and heptamer sequences
Predicted secondary structure of single-stranded target	Abundant	Less abundant	?
Immunoglobulin enhancer sequence	Dispensable	Required?	?
NHEJ repair	Required	Dispensable	Required

STAGGERED NICK
A form of DNA double-stranded break that produces 3′- or 5′-protruding ends.

ERROR-PRONE REPAIR
A general term for the repair of various types of DNA lesion at the expense of fidelity.

Because cleaved ends are joined by the NHEJ systems during class-switch recombination, single-stranded tails of staggered ends might be blunt-ended by exonucleases and might involve error-prone DNA polymerases. During these processes, frequent mutations are introduced in the region surrounding the recombination breakpoints[44,45] (FIG. 4b).

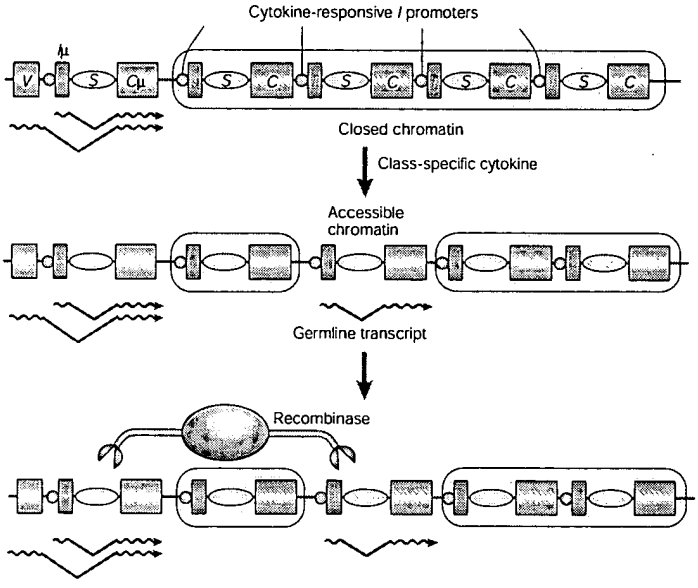


Figure 2 | **Accessibility model of class-switch recombination.** In resting B cells, the μ chain mRNA encoding the *V* and *C*μ (green) regions is transcribed from the immunoglobulin heavy-chain locus. In addition, germline transcripts are synthesized from the intron promoter (Iμ) located 5′ to the *S*μ region. The Iμ exon is spliced to the *C*μ exons in mature germline transcripts, which do not code for any proteins. Downstream *S* and *C* regions in the shaded area are in the transcriptionally inactive chromatin. Cytokine stimulation activates transcription from a specific intron promoter located 5′ to the *S* region and induces chromatin opening as indicated by a gap in the shaded area. Transcriptionally active *S* regions are considered to be accessible to the class-switch recombinase. Red waves with an arrowhead represent exon sequences of transcripts and connecting v-shaped lines indicate splicing.

Molecular basis of somatic hypermutation

Somatic hypermutation introduces point mutations specifically in immunoglobulin *V* genes of activated B cells at an extraordinarily high frequency (10^{-3} bases per generation)[46,47]. This frequency is roughly one million times higher than the level of mutation during normal DNA replication (10^{-9} bases per generation). Not all IgG or IgA undergo somatic hypermutation, and conversely, some IgM have somatic hypermutation, indicating that neither class-switch recombination nor somatic hypermutation is a prerequisite of the other[48,49].

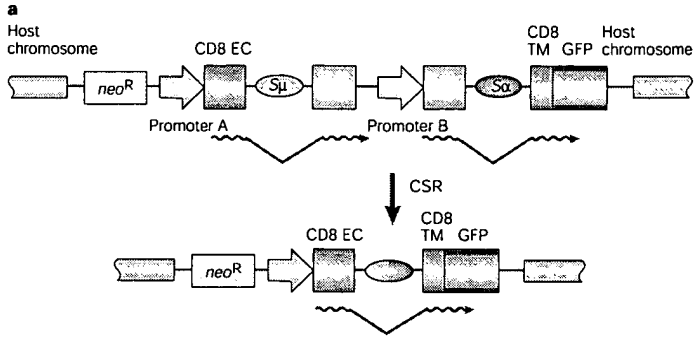
Selection and recognition of the target. Transcription seems to be essential for somatic hypermutation. Mutations are commonly found within a 1.5–2-kb region that lies 3′ to the promoter region of the rearranged *V* gene, regardless of families and endogenous or transgenic loci, and mutations upstream of the transcription start site are extremely rare. Moreover, the intronic enhancer and promoter have been shown to be required for somatic hypermutation[50,51]. However, immunoglobulin promoters can be replaced with heterologous promoters, to which either RNA polymerase II or I can bind[51]. Because the mutation rate is positively correlated with the transcription efficiency[51,52], it should be carefully examined to find out whether the enhancer sequence is essential for functions other than transcriptional activation, as proposed[53]. Note that the transcription-inducible construct used in the more recent work does not contain an enhancer[52].

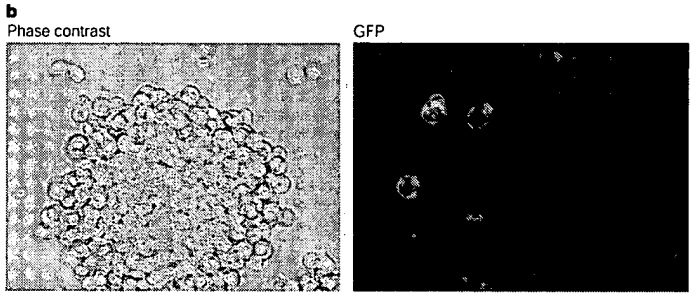
Since Brenner and Milstein[54] indicated the involvement of DNA cleavage (probably a nick) and ERROR-PRONE REPAIR in somatic hypermutation, several studies have aimed to identify the recognition target by a putative mutator or nicking enzyme, the mode of cleavage, and the enzymes responsible for error-prone repair. Intrinsic hotspots of the mutation ('hypervariable regions') overlap the COMPLEMENTARITY-DETERMINING REGIONS (CDRs), which directly contact antigens. The TAA and RGYW motifs (where R = G or A; Y = T or C; W = A or T) are statistically preferred hotspots for mutation[55]. However, because the mutation can occur outside the TAA and RGYW motifs, and because these motifs *per se* are not always mutated, it is not clear whether these motifs are the recognition target. Transgenic experiments showed that somatic hypermutation can occur in various non-*V* gene sequences, including the coding sequences of chloramphenicol acyltransferase[56], β-globin, neomycin phosphotransferase, guanosine phosphoribosyl transferase[57] and the immunoglobulin *C*κ region[58]. In addition, introns in the human *BCL-6* gene are somatically mutated, albeit much less frequently[59,60]. Furthermore, a completely artificial sequence containing alternating *Eco*RV and *Pvu*II restriction sites inserted into an *Igk* transgene is a hotspot for mutations[61]. Therefore, as in the case of class-switch recombination, somatic hypermutation does not seem to have a clear primary-sequence specificity.

Storb and colleagues[62] proposed that transcription is required to recruit a putative mutator (possibly a

COMPLEMENTARITY-DETERMINING REGION (CDR). Segment in the variable region genes of immunoglobulins and T-cell receptors, corresponding to loops of polypeptide chains that shape the complementary surface to the contour of antigens.

'nickase'). According to their model, the mutator can bind to an RNA polymerase II during initiation of transcription, and it is transferred to DNA when the RNA polymerase pauses by a secondary structure (stem–loop) of the nascent transcript. The stem–loop-forming sequences are found not only in the *Eco*RV/*Pvu*II sequences mentioned above, but also, to varying degrees, in any *V* genes[63] or in the other sequences that have been shown to be subject to somatic hypermutation in experimental systems[56–58,61].





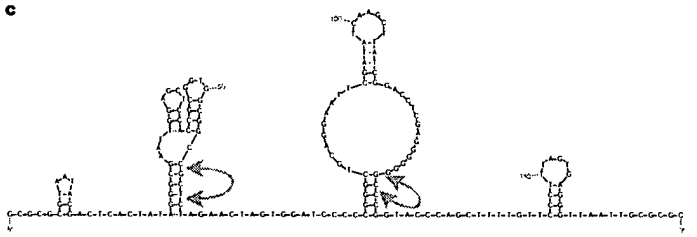


Figure 3 | **Artificial substrates of class-switch recombination. a** | The basic structure of an artificial substrate described in the text is shown. It contains two *S* regions transcribed by separate constitutive promoters. *S* sequences are removed by splicing from transcripts produced by both promoters. This substrate contains the neomycin-resistance gene (*neo*^{R}), which allows stable transfection into cell lines. The upstream and downstream transcription units contain the coding sequence of the extracellular (EC) domain of CD8α and the sequence for the transmembrane (TM) domain of CD8α fused with green fluorescent protein (GFP), respectively. Before class-switch recombination, stable transfectants express GFP in the cytoplasm. When deletion occurs between the two *S* regions by class-switch recombination, the CD8α–GFP fusion protein is expressed on the cell surface, which is detectable by fluorescence-activated cell sorting (FACS) and direct microscopy as shown in **b**, where only switched cells express GFP on the surface. **c** | Computer-predicted secondary structure of an artificial sequence containing multiple cloning sites from a conventional plasmid vector. Owing to the presence of palindromic sequences, the stem–loop structure may be formed from the single-stranded DNA. Arrowheads indicate actual class-switch recombination junctions generated in the artificial substrate[40].

Kolchanov and colleagues[63] speculated that the mutator might recognize non-complementary base pairs in the stem–loop structure of the *V*-region genes because the abundance of complementary palindromes in *V*-region genes is about four times higher than that in other genes including immunoglobulin *C*-region genes.

Cleavage. Although most models of somatic hypermutation propose a break of DNA strand(s), direct evidence to support DNA cleavage was obtained only recently[53,64–68]. Deletions, duplications or insertions were found in *V* genes of germinal-centre B cells[64,65]. The idea that DNA breaks occur during somatic hypermutation is supported by the observation[66] that short, non-templated additional bases are inserted into a constitutively mutating *V* gene in a human B-cell line (Ramos) overexpressing TERMINAL DEOXYRIBONUCLEOTIDE TRANSFERASE (TdT). Two other groups detected double-stranded lesions in *V* genes of B cells undergoing somatic hypermutation[53,67].

Repair. The final step of somatic hypermutation — repair of the cleaved ends — should be the key step for introducing point mutations. Many groups have explored the possible involvement of repair enzymes using gene targeting, but the results are a bit confusing. Whereas the deficiency of some proteins showed no effect[69,70], deficiency of mismatch repair proteins including Pms2 (REFS 71–74), Msh2 (REFS 73–76) and Mlh1 (REF. 77) caused lower mutation frequencies and/or altered mutation spectra. And interpretation of all available results has led to two opposing views: either that mismatch repair modifies or fixes incipient mutations; or that mismatch repair has no role in somatic hypermutation. However, it is worth noting that Msh2 deficiency affects the efficiency and local sequence specificity of both class-switch recombination and somatic hypermutation[75,78–80]. Recently, homologous recombination with the sister chromatid was proposed to be involved in the repair of double-stranded DNA breaks and in the generation of mutations[53]. Homologous recombination has also been proposed as an explanation for gene conversion in avian *V* genes[81,82]. One clear finding, however, is that the NHEJ system is dispensable for somatic hypermutation[83].

The requirement for AID

Class-switch recombination depends on *de novo* protein synthesis, so we did complementary DNA subtraction between switch-induced and non-induced CH12F3-2 cells to identify either a class-switch recombinase or its activator[21]. We isolated *AID*, which is expressed specifically in B cells that have been activated *in vivo* or *in vitro*. *In situ* hybridization showed that *AID* expression is restricted to germinal centres, where class-switch recombination and somatic hypermutation take place.

Overexpression of *AID* in CH12F3-2 B cells enhanced class-switch recombination, irrespective of cytokine stimulation. Mice deficient for *AID* cannot produce IgG, IgA or IgE antibodies, whereas IgM is normally (or even more abundantly) produced under

TERMINAL DEOXYRIBONUCLEOTIDE TRANSFERASE (TdT). An enzyme that adds non-templated nucleotides to a 3'-hydroxyl end of DNA.

LIPOPOLYSACCHARIDE (LPS). A component of the outer wall of Gram-negative bacteria, consisting of a sugar chain and lipid adducts.

CHYLOMICRON A lipoprotein involved in transfer of the lipids absorbed in the small intestine.

immunized or non-immunized conditions[5]. Immunoglobulin classes other than IgM and IgD were not detected, even after LIPOPOLYSACCHARIDE (LPS) and cytokine stimulation of spleen cells *in vitro*. Sequence analysis of the *VH186.2 V* gene in *AID*$^{-/-}$mice after immunization with 4-hydroxy-3-nitrophenylacetyl (NP) conjugated with chicken γ-globulin (CGG) showed that the mutation frequency was no more than the error rate of the Taq polymerase used for the experiment.

A mutation in the *CD40L* gene has been shown to cause a condition called X-linked hyper-IgM syndrome type 1 (HIGM1), which manifests severe immunodeficiency owing to a defect in class-switch recombination[84–88]. There is also another type of HIGM (HIGM2), which shows similar clinical symptoms but is inherited in an autosomal-recessive manner[89]. Both the human *AID* gene and the *HIGM2* locus have been mapped to chromosome 12p13, and sequencing of the *AID* coding region from 18 patients with HIGM2 identified various mutant alleles, all of which would give rise to mutant AID proteins with amino-acid replacements or truncations[90]. Moreover, somatic hypermutation was not observed in B cells from HIGM2 patients. These findings in human and mouse convincingly demonstrate the requirement of AID for both class-switch recombination and somatic hypermutation.

In vitro stimulation of B cells from *AID*-deficient mice with LPS and cytokines induced normal germline transcription of downstream *S* regions. This implies that AID is involved in neither signal transduction from the cell-surface receptor to the nucleus, nor in the establishment of the accessible state of the *S* region. *V(D)J* recombination is normal in humans and mice with the *AID* mutation, indicating that AID is not involved in the repair process that is shared between *V(D)J* recombination and class-switch recombination. In other words, AID is probably involved in the cleavage step during class-switch recombination.

The predicted amino-acid sequence (198 residues) of AID has homology to that of APOBEC-1, which is a catalytic subunit of the RNA-editing complex for the apolipoprotein B messenger RNA precursor[91]. APOBEC-1 converts the specific cytosine at position 6666 of apolipoprotein B mRNA to uracil by its intrinsic cytidine deaminase activity, giving rise to mRNA that encodes the CHYLOMICRON component. Although APOBEC-1 cannot bind a specific sequence in the target mRNA, this specificity is conferred by a protein with which it associates, APOBEC-1 COMPLEMENTATION FACTOR (ACF)[92]. Like APOBEC-1, AID synthesized *in vitro* has cytidine deaminase activity[21]. Moreover, both the *APOBEC-1* and *AID* genes are closely mapped to human chromosome 12p13. These structural, genetic and functional relationships of AID and APOBEC-1 indicate that AID might be an RNA-editing enzyme. AID, in a complex with an ACF-like protein, might edit a certain mRNA, giving rise to a new mRNA that encodes the class-switch recombinase.

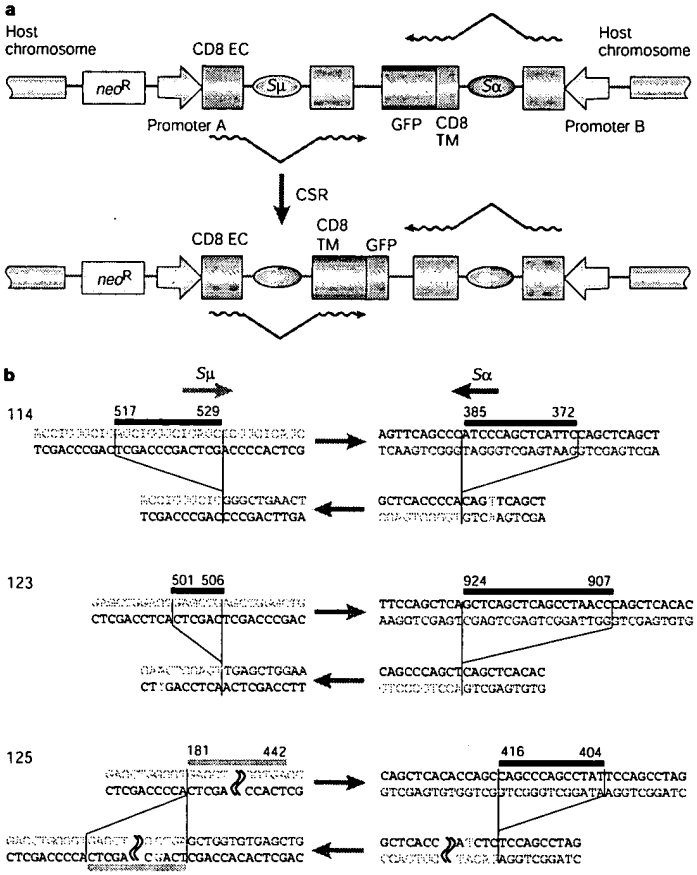


Figure 4 | **Inversions and deletions at class-switch recombination junctions. a** | A variant of the class-switch recombination substrate, in which two transcription units are placed in an opposite orientations (facing each other) is shown. Inversion-type class-switch recombination is observed after stimulation[43]. **b** | Junction sequences in three independent clones (114, 123 and 125) of CH12F3-2 transfectants, with the substrate shown above. In each set, the top and bottom sections show double-stranded sequences before and after class-switch recombination. Junctions are represented by vertical lines. Black arrows represent the orientation of segments between junctions. The non-template strands of Sμ and Sα are orange and maroon, respectively, whereas the template strand is marked with black letters. Mutations are marked with light blue letters. Black and maroon horizontal bars indicate the range of deletions and duplication, respectively. Numbers indicate positions from the promoter-proximal end of the *S* regions. (EC, extracellular domain; TM, transmembrane domain.)

A common mechanism?

Class-switch recombination and somatic hypermutation share many important features, although their targets and products are different (TABLE 2). The most striking common features for the two processes are the requirements for AID and target transcription, and the involvement of DNA cleavage. As described above, the stem–loop structure is speculated to be the recognition target for both class-switch recombination and somatic hypermutation. Because the RNA polymerase holoenzyme complex contains a DNA HELICASE component, the DNA strands in the proximity of actively transcribing RNA polymerase are thought to be transiently denatured. The secondary structure in the single-stranded DNA may then persist long enough to be recognized by the recombinase or mutator (FIG. 5a). Another possible

APOBEC-1 COMPLEMENTATION FACTOR (ACF)
An RNA binding subunit of an apolipoprotein B mRNA editing complex (editosome) that is required for conversion of mRNAs for the low-density lipoprotein component, ApoB100, to those for the chylomicron component, ApoB48.

DNA HELICASE
An enzyme that unwinds duplex DNA during replication, transcription, repair and recombination.

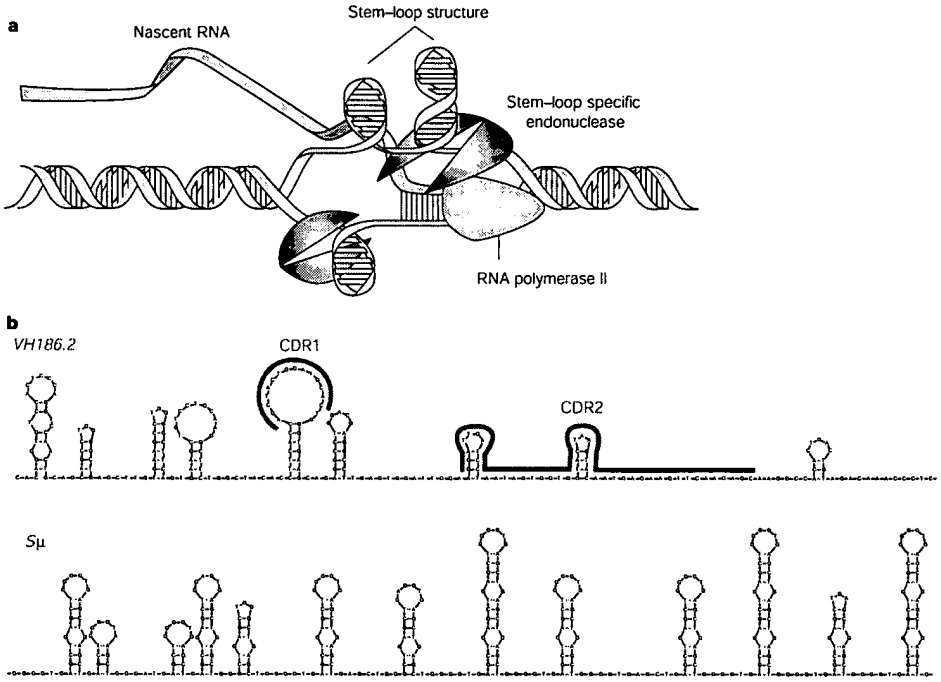


Figure 5 | **Structure of the target DNA in class-switch recombination and somatic hypermutation. a** | Schematic for generation of the secondary structure during transcription by RNA polymerase II (red oval) and its recognition by putative stem–loop-specific endonucleases (blue). DNA and RNA are represented by blue and green ribbons, respectively. **b** | Computer-predicted secondary structure of a V_H segment (VH186.2) and a part of mouse Sμ sequence (only one-tenth). Sequences corresponding to CDR1 and CDR2 of VH186.2 are marked by red lines.

secondary structure may be an RNA/DNA heteroduplex (R loops) that can also transiently form over *S* regions during transcription[68,93].

On the basis of this speculation, we propose a model in which the endonuclease(s) encoded by AID-edited mRNA has recognition specificity to the stem–loop structure, and cleaves in its proximity in the *V* and *S* regions during somatic hypermutation and class-switch recombination reactions, respectively. This model (FIG. 6) postulates that transcription of *S* regions might be required not only for opening the chromatin structure, but also to form the secondary structure that will be recognized by the endonuclease. Because such recognition and cleavage take place separately on each strand, cleaved ends show either a single-stranded nick or double-stranded staggered cleavage. In the case of class-switch recombination, single-stranded tails of staggered cleavage ends must be either filled or chewed to form blunt-ends before being repaired by the NHEJ system. This may be carried

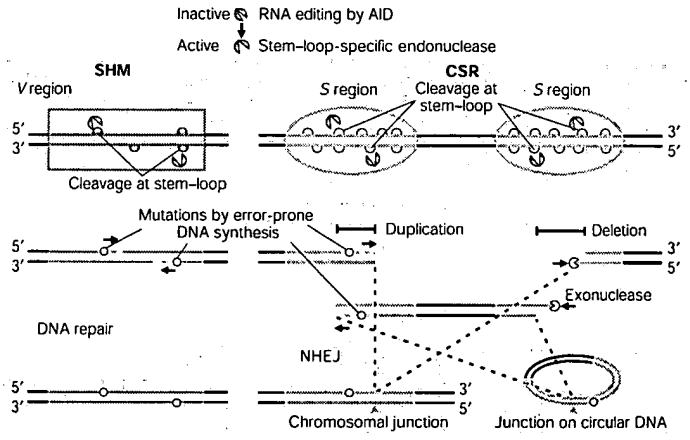


Figure 6 | **Model for the regulation of class-switch recombination and somatic hypermutation by AID.** *V* and *S* regions are indicated by a red rectangle and green oval, respectively, superimposed with their predicted secondary structures. CD40L and cytokines induce expression of *AID*, which probably edits messenger RNA for an inactive enzyme to convert it to an RNA for an active endonuclease specific to stem–loop structures (open scissors). Nicking of *S*-region DNA occurs frequently at different positions on both strands of DNA, giving rise to double-stranded cleavages with staggered ends. Nicking of *V*-region DNA is less frequent, generating a mixture of single- and double-stranded breaks. Broken ends in *S* regions are repaired by the non-homologous end-joining (NHEJ) pathway, whereas those in *V* regions are repaired by an unknown mechanism other than NHEJ. Both processes involve digestion by exonuclease(s) (yellow pacman) and DNA synthesis by error-prone polymerase(s), giving rise to mutations (yellow circle) near cleavage sites.

out by exonucleases and/or error-prone DNA polymerases, which introduce mutations near the cleaved and ligated junctions[44,45].

Conversely, in the case of somatic hypermutation, the *V* gene might be attacked by the same or similar endonucleases, but less efficiently, probably owing to the less extensive secondary structure (FIG. 5b). This results in the generation of single-stranded nicks, which could be repaired by low-fidelity DNA polymerases, exonuclease and ligase. Mutations are most likely to be introduced during this repair. In some cases, AID-induced cleavage in *V* genes also takes place on two strands, generating double-stranded staggered ends, the single-stranded tails of which might be processed as described for class-switch recombination. Although recent reports[53,67] indicate the presence of double-stranded blunt ends in the *V* gene of the hypermutating B cells, nick and/or staggered cleavages could be primary products by the mutator[47,94]. It is well established that the repair systems for class-switch recombination and somatic hypermutation are distinct[22–24,83].

Future problems

The involvement of AID in class-switch recombination as well as in somatic hypermutation has added another layer of complexity to our understanding of the immune defence system in vertebrates. These findings also indicate that a wider range of biological regulations than previously thought might depend on RNA editing. At this stage, however, our knowledge of the functions of AID is limited. Obvious questions are: is AID really the RNA-editing enzyme? If so, what is the target mRNA? And are there any cofactors for AID, like ACF for APOBEC-1?

As many genes can form transient stem–loop structures during transcription, albeit less abundantly, it is possible that some additional regulation mechanism might be involved to discriminate immunoglobulin *V* genes and *S* regions from non-immunoglobulin genes. Although immunoglobulin enhancers and/or their binding proteins have been suggested to be a part of the locus-recognition sequence that initiates somatic hypermutation, we now need to address whether the dependence on enhancers is due solely to the efficiency of transcription. Our recent data indicate that the efficiency of transcription also correlates with that of class-switch recombination (C.-G. Lee, K.K. and T.H., unpublished data). In addition, there might be a mechanism to regulate class-switch recombination and somatic hypermutation separately, because the two events occur independently.

It is also important to know what types of error-prone DNA polymerase are involved in somatic hypermutation as well as in class-switch recombination. The ligation system of repaired cleaved ends in somatic hypermutation is another important question. It has been speculated that B-cell malignancy associated with chromosomal translocation involving immunoglobulin genes could be by-products of class-switch recombination or somatic hypermutation[64,95]. This possibility will be tested directly using *AID*$^{-/-}$ mice. Answers to all these questions will eventually advance our knowledge of dynamic regulation of the genomic information by genetic alteration, which might also be involved in complex biological phenomena other than the immune system.

Links

DATABASE LINKS RAG-1 | RAG-2 | IgM | IgD | IgE | AID | CD40 | IL-4 | CD40L | TGF-β | *BCL-6* | Pms2 | Msh2 | Mlh1 | HIGM1 | HIGM2 | APOBEC-1 | ACF | Adar2 | Adar1

FURTHER INFORMATION Computer program | Honjo lab

ENCYCLOPEDIA OF LIFE SCIENCES Somatic hypermutation in antibody evolution | Germinal centres

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Brief Definitive Report

The Activation-induced Deaminase Functions in a Postcleavage Step of the Somatic Hypermutation Process

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Abstract

Activation of B cells by antigen fuels two distinct molecular modifications of immunoglobulin (Ig) genes. Class-switch recombination (CSR) replaces the Igμ heavy chain constant region with a downstream constant region gene, thereby altering the effector function of the resulting antibodies. Somatic hypermutation (SHM) introduces point mutations into the variable regions of Ig genes, thereby changing the affinity of antibody for antigen. Mechanistic overlap between the two reactions has been suggested by the finding that both require the activation-induced cytidine deaminase (AID). It has been proposed that AID initiates both CSR and SHM by activating a common nuclease. Here we provide evidence that cells lacking AID, or expressing a dominant negative form of the protein, are still able to incur DNA lesions in SHM target sequences. The results indicate that an intact cytidine deaminase motif is required for AID function, and that AID acts downstream of the initial DNA lesions in SHM.

Key words: somatic hypermutation • AID • DNA double-strand breaks • B lymphocyte • Ig gene

Introduction

One of the hallmarks of the immune system is the ability to recognize large numbers of antigens. In the mouse and human B cell repertoires, diversity is generated by two distinct processes; the primary repertoire is generated in the bone marrow by V(D)J recombination (1). Its further diversification involves the antigen-driven introduction of point mutations into the V regions of Ig genes (1). This process of somatic hypermutation (SHM) gives rise to families of related mutant antibodies which are then selected for their binding affinity to the immunizing antigen (2).

During hypermutation, the V regions of Ig genes accumulate single nucleotide substitutions and occasional insertions and deletions. Many of the mutations occur at specific residues (hotspots), suggesting that the sequence surrounding a hotspot might be the target of a putative mutator complex (3). Yet whenever the V gene has been replaced with artificial substrates, those substrates hypermutate successfully (3), and so the sequence of the V gene itself does not initiate the mutation process. Surprisingly, the specific V region promoter can be replaced by heterologous promoters without a detrimental effect on hypermutation (3), but the process is absolutely dependent on the presence of the Ig enhancers. Because of this reliance on promoter and enhancer elements, it is has been suggested that the targeting step of hypermutation is linked to transcription, although whether it is coupled to the transcription process per se or to a general requirement for locus accessibility remains unclear (4–6).

SHM was originally hypothesized to be a two-step process, initiated by cleavage of the DNA within the mutating region, and subsequently resolved by error-prone repair (7). There is strong circumstantial evidence to suggest that the cleaved DNA intermediate is a DNA double-strand break, as V regions of hypermutating cells incur DSBs on hotspots and at high rates (8, 9). Furthermore, the initiation of DSB formation has been shown to require the same elements which regulate the introduction of mutations (transcription and the presence of the enhancer) (8, 9). These hypermutation-associated DSBs are abundant in the G2 (postreplicative) phase of the cell cycle, and thus it has been suggested that they are repaired by homologous recombination between sister chromatids (8).

Even though it has been extensively studied, little is known about the molecular mechanism of the SHM process. The only known protein whose loss of function leads to a significant downregulation (if not total ablation) of hypermutation is the recently discovered activation-induced cytidine deaminase (AID) (10, 11). Yet, the function of

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AID in somatic hypermutation is far from clear, and its effect might not even be direct, as AID is thought to be an RNA-editing enzyme (12). It has been postulated that AID edits the mRNA of the endonuclease responsible for the DNA lesions in both CSR and SHM (10). Alternatively, AID might be responsible in editing the mRNA of a factor (or factors) responsible for orchestrating DSB repair. Here we report that SHM-associated DSBs are still present in cells lacking AID, or expressing a dominant negative form of the protein. Our results indicate that AID acts downstream of the initial DNA lesions in SHM, possibly by editing the RNA of a repair factor.

Materials and Methods

Plasmid Constructs. The AID cDNA was amplified from Ramos cDNA and the H56R/E58Q mutations in AID were introduced with the QuikChange™ kit (Stratagene). To generate the bacterial expression vectors, AID or AID DN were independently cloned into pET3d in frame with GST, to generate the wild-type GST-AID or GST-AID DN fusion gene, respectively. The proteins were expressed in BL21 λDE3 cells and purified with glutathione sepharose 4B resin, according to the manufacturer's instruction (Amersham Pharmacia Biotech).

To generate the retroviral expression vectors, the AID DN cDNA was cloned into the pMSCV retroviral expression vector (CLONTECH), engineered to express either GFP or a puromycin resistance gene from an internal phosphoglycerol kinase (pgk) promoter.

Mice and Cell Culture. $AID^{-/-}$ (10) mice have been described previously. The Ramos (RA-1) cell line was obtained from the American Type Culture Collection and grown as described previously (13). For retroviral infections, pMSCV or pMSCV-AID DN, along with the packaging plasmid pkat (14), were cotransfected into 293T cells by calcium phosphate precipitation and viral supernatants harvested 48 h after transfection were used to infect cells.

Deamination Assay. The deamination assay was performed as described previously (15). Briefly, 100 μg of protein was added to a mix containing 25 μmol Tris-HCl, pH 7.05, and 1 μmole cytidine (1 ml final volume). At different times, 100 μl samples were added to 900 μl of 0.5 M ice-cold perchloric acid and the mixture was centrifuged. The absorbance of the supernatant was measured at 290 nm. Under these conditions, the difference in the molar extinction coefficient between cytidine and uridine is 10.1×10^6 cm^2 per mol (15).

LM-PCR and RT-PCR. Cells were embedded in agarose and genomic DNA prepared as described previously (8). The sequences of gene specific primers not described in (8) were as follows: mouse VH186.2, VH186.2 L1 TTCTTGGCAGCAACAGCTACAGGTAAGG VH186.2 L2 GCAGGCTTGAGGTCTGGACATATACATG probe GACATCCACTTTGCCTTTCTCTCCACAGGTG; mouse Vλ1, Vλ1 L1 GTTGTGACTCAGGAATCTGCACTCACC Vλ1 L2 GGTGAAACAGTCACACTCACTTGTCGC probe GTACTGGGGCTGCTACAACTAGTAACTATGCCAACTG; and mouse Cμ, Cμ L1 GCTTCCCATCAGTCCTGAGAG Cμ L2 CCACCTCACAGGTGCTGCTGCCTTCC probe CACGTGGTGTGCAAAGTCCAGCACC. Total Ramos RNA was prepared using RNAsol (Tel-Test, Inc.), treated with DNase, and reverse transcribed using random nonamers. c-myc, and human AID were PCR amplified (30 cycles) from cDNA from 10^5 cells using hotstart Taq (QIAGEN) and combinations of the following primers: human AID, hAID left GTCCGCTGGGCTAAGGGTCGGCG hAID right GCACCCCGGCGCGGTGCAGCCG; and human c-myc, c-myc left CCGCCCGCGGCCACAGCGTC c-myc right CGCCTCTTGACATTCTCCTCGGTGTC.

Flow Cytometry and Cell Sorting. To purify germinal center B cells, we pooled spleens from mice at 14 d after immunization with NP-CGG in alum (Biosearch Technologies, Inc.), and separated the resting and activated lymphocytes on a Percoll gradient (Amersham Pharmacia Biotech). Cells were stained with the pan-B cell marker B220 (phycoerythrin) and the B/T activated cell marker GL-7 (FITC) (both from BD PharMingen), and sorted on a Becton Dickinson FACSVantage™. Sorted cells were immediately embedded in agarose for further analysis. For the cell cycle study, standard methods were used (16). All analyses of flow cytometric data were performed using the FlowJo™ software package (Treestar).

Results

An AID Catalytic Mutant Has No Deaminase Activity. The primary sequence of AID shares highest sequence similarity with Apobec-1 (12) (Fig. 1 A), the prototypic RNA-editing cytidine deaminase (14). Cytidine deaminases in general share a conserved active site motif consisting of a histidine and two cysteines which coordinate Zn^{2+}, and a glutamate which serves as a proton donor in the deamination reaction (17). Replacement of any of these four amino acids results in a catalytic mutant (18). We generated an AID protein mutated in two of these residues (H56R/E58Q) and expressed both the mutant and wild-type proteins in *Escherichia coli.* The wild-type protein was active in an in vitro deamination assay while the catalytic mutant was not (Fig. 1 B).

All except one of the known cytidine deaminases act as homodimers or homotetramers (19), and hence catalytic mutants can have a dominant negative effect (19, 20). A series of leucines in the carboxy terminus of Apobec-1 is thought to be important for dimerization (20), and even though there is low sequence homology between AID and Apobec-1 in that region, these leucines are conserved in AID. Furthermore, secondary structure prediction algorithms (21) model the carboxy terminus as almost entirely α-helical with a potential for coiled coil formation (Fig. 1 C). Hence the H56R/E58Q mutant AID (henceforth AID DN) might display a dominant negative phenotype.

AID DN Interferes with SHM. To determine the effect of the putative dominant negative protein on SHM, we expressed the protein in the human Burkitt lymphoma cell line Ramos, which hypermutates constitutively in culture (13). Cells were infected with a retrovirus expressing human AID DN from the long terminal repeat promoter and a puromycin resistance gene (puro) from an internal pgk promoter. Infected cells selected with puromycin expressed AID DN at levels 20-fold higher than endogenous AID, as assessed by semiquantitative RT-PCR (Fig. 2 A). As controls, cells were infected with retroviruses expressing only the puro gene (designated "empty") or wild-type AID in addition to puro. Infected cells (puromycin resistant) were

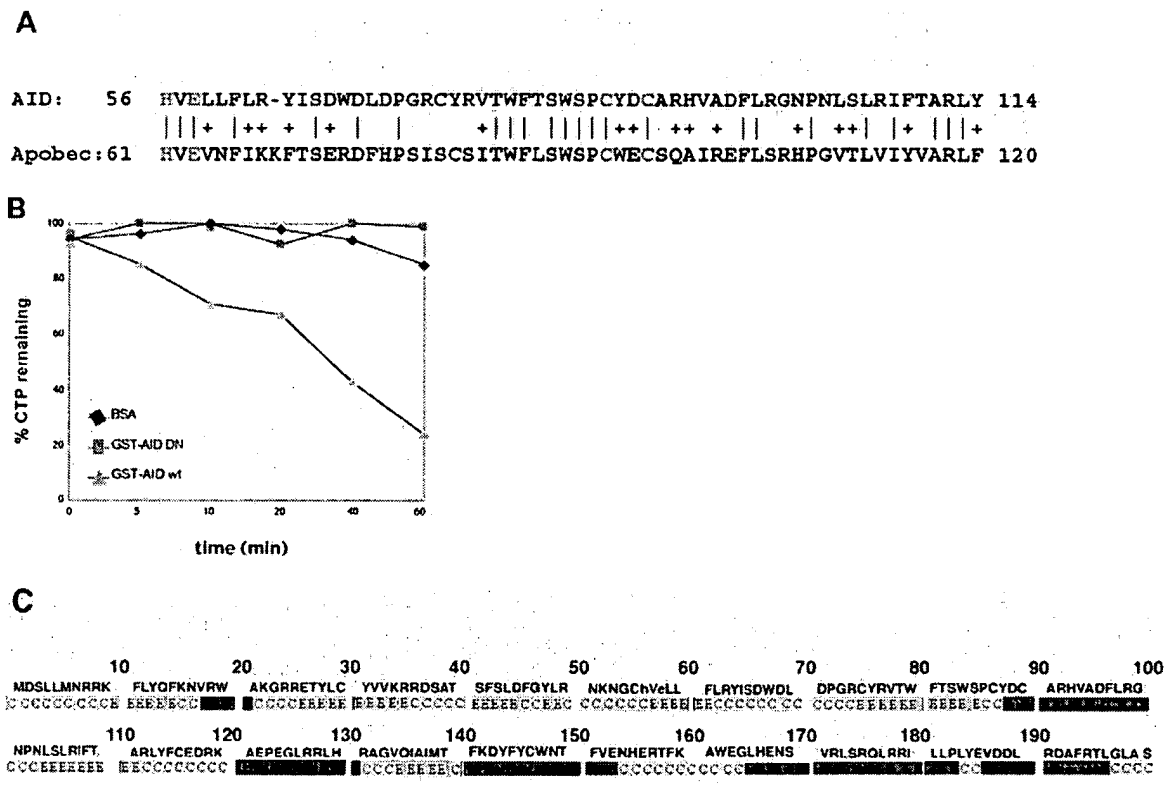

Figure 1. Characterization of recombinant AID proteins. (A) Alignment of the predicted active site of AID and the active site of Apobec-1. Conserved catalytic residues are indicated in red. (B) Cytidine deamination assay. Recombinant GST-AID wild-type (WT) and GST-AID DN mutant were expressed in and purified from bacteria. CTP was incubated with purified GST-AID WT, GST-AID DN, or BSA, and the amount of substrate remaining at different time points was determined by absorption at 290 nm. The experiment was repeated with AID DN tagged with a minimal tag (strep tag, Cell Genesys) with similar results (data not shown). (C) Secondary structure prediction for human AID. C (gray) indicates potential loops (or nonstructured regions), E (yellow) indicates a β-sheet, and H (blue) indicates an α-helix (modeling was done on the 3D-PSSM server; reference 21).

seeded at single cell density, and after 6 wk of expansion, the Ig heavy chain variable region was PCR amplified, cloned, and sequenced. We assayed six clones infected with the empty retrovirus, and in all cases, 4–6 independent mutations were identified (Fig. 2 B), yielding a mutation frequency of 0.5×10^{-5}/bp/generation (calculated as per Capizzi and Jameson; for a review, see reference 22). The results from the analysis of clones infected with wild-type AID retrovirus were comparable (data not shown). In contrast, Ramos clones expressing AID DN contained virtually no mutations (Fig. 2 B), yielding a mutation frequency at least 20-fold lower than that of cells infected with the empty virus (0.02×10^{-5}/bp/generation). We conclude that expression of AID DN suppresses SHM (presumably by interfering with the function of the wild-type protein), yielding a phenotype functionally equivalent to full AID deficiency. The results also indicate that an intact cytidine deaminase catalytic motif (and therefore deaminase activity) is essential for the function of AID.

SHM-associated DSBs Are Not Affected by Expression of AID DN. Ramos cells were infected with retroviruses that expressed either puromycin alone (empty virus), or puromycin in conjunction with wild-type AID or AID DN, selected in the antibiotic, and assayed for DSBs in the Ig heavy and light chain variable regions. Consistent with our previous report (8), we can titrate the number of linker-ligated genomes down to 50 and still detect DSBs in Ramos cells infected with the empty virus or with the wild-type AID virus (Fig. 3 A and data not shown). Ramos cells infected with the AID DN retrovirus continue to in-

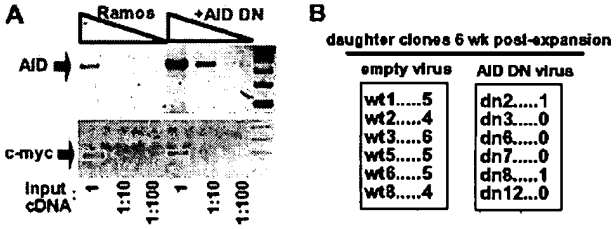

Figure 2. AID DN interferes with SHM. (A) RT-PCR analysis of AID expression levels in wild-type Ramos cells (left) and Ramos cells transduced with an AID-DN–expressing retrovirus (right). Amplification of c-myc controls for input cDNA. (B) IgH variable region mutations per culture after 6 wk of expansion (based on 48 sequences per culture). Cultures were grown from single Ramos cells transduced with empty virus or with AID-DN–expressing virus.

cur DSBs over the Ig heavy variable region, at a similar or even slightly higher frequency than uninfected cells or those infected with the empty virus (bands are reproducibly detected with as few as 10 input genomes; Fig. 3 A and data not shown). Similar results were obtained for the Igλ variable region (data not shown). Thus, interference with AID function through expression of AID DN does not diminish the amount of DSBs over the Ramos variable regions, even though it dramatically reduces mutation accumulation.

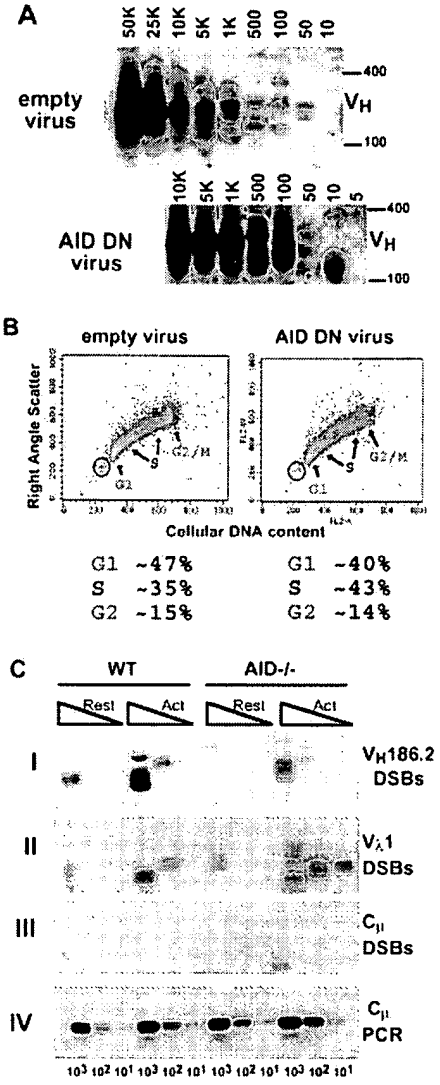

Figure 3. SHM-associated DSBs are not abrogated by AID DN or AID deficiency. (A) LM-PCR amplification of DSBs from the endogenous V_H region of Ramos cells infected with empty virus or with AID DN virus. Genome equivalents of linker-ligated template DNA are indicated above the lanes, and the size of molecular weight markers to the right (bp). (B) Cell cycle status analysis of retrovirally infected Ramos cells using propidium iodide staining. The percentage of cells in G1, S, and G2 are indicated below the FACS® plots. The circle indicates the expected location of apoptotic cells. (C) LM-PCR amplification of DSBs from the V_H186.2, Vλ1, and Cμ genes from C57BL/6 (WT) or AID$^{-/-}$ mice 14 d after immunization with NP-CGG. Resting (B220$^+$, GL7$^-$, small) and activated (B220$^+$,GL7$^+$, large) splenic B cells were compared. Genome equivalents of linker-ligated template DNA are indicated below the lanes. Standard PCR of Cμ (bottom panel) indicates that equivalent amounts of DNA were used for each PCR reaction.

DNA DSBs can be lethal to the cell if not repaired. Since the introduction of mutations is thought to occur during the repair of SHM-associated DSBs (8, 9), it was possible that Ramos cells expressing AID DN (which have abundant DSBs but very few mutations) would exhibit substantial cell death. To investigate this possibility, we stained Ramos cells with propidium iodide and analyzed them for DNA content. The percentage of cells in the G1, S, and G2 phases of the cell cycle did not vary significantly between Ramos cells infected with empty virus, wild-type AID or the AID DN virus (Fig. 3 B and data not shown). Importantly, no subdiploid peak indicative of dead cells was observed in any of the cultures. Furthermore, neither the proliferation rate nor the restriction of DSBs to the late S/G2 phase of the cell cycle (8) was affected by AID DN expression (data not shown). Thus, Ramos cells infected with the AID DN retrovirus do not die as a result of unresolved DSBs, indicating that these DSBs are repaired but predominantly in an error-free fashion.

SHM-associated DSBs Are Not Affected by Deletion of AID. Our data suggest that ablation of the catalytic activity of AID results in a dominant negative phenotype with respect to SHM, but does not impair the formation of SHM-associated DSBs. It is possible, however, that AID has functions that do not depend on the cytidine deaminase activity (for instance in facilitating DSB formation). To examine this possibility, we assessed Ig variable region DSBs in activated B cells lacking AID. Wild-type and AID$^{-/-}$ C57BL/6 mice were immunized with NP-CGG, an antigen preferentially recognized by a specific heavy and light chain combination (V_H186.2 and Vλ1) (23). At 14 d after immunization, splenic B cells were collected and sorted into germinal center (GC) and resting B cell populations. We could reproducibly amplify DSBs over the V_H186.2 and Vλ1 genes both from wild-type and AID$^{-/-}$ cells (Fig. 3 C and data not shown). Such DSBs could be amplified from both the GC and the resting B cell fractions, but were preferentially present in the GC B cell population (10- to 100-fold higher frequency, Fig. 3 C, panels i and ii). The observed DSBs were V region specific since no DSBs could be amplified from the Ig μ constant region or from the recombination activating gene (RAG)-1 gene (Fig. 3 C panel iii, and data not shown). We conclude that in the absence of AID, SHM-associated DSB formation remains intact both in vitro and in vivo, and therefore, that AID likely functions in a postcleavage step of the SHM process.

Discussion

The immune system generates diversity by using three distinct mechanisms of genetic alteration. V(D)J recombination, CSR, and SHM. Mechanistically, all three processes can be divided into at least three phases: targeting/

recognition, DNA cleavage, and repair (18). In all three cases, transcription is thought to play a key role in targeting of a nuclease to the Ig locus. The cleavage step is thought to result in the production of a DNA DSB, though the creation of the DSB might not be the initial event. In V(D)J recombination for instance, the initial event is the nicking of one strand by the RAG recombinase, followed by hairpin formation and resolution into a blunt DSB (24). It is not known whether the CSR nuclease initially inflicts a single or double-strand break but there is some evidence to suggest that the final product of cleavage is a DSB (25). Finally, there is data to indicate that SHM also involves the creation of a DNA DSB, though that may not necessarily be the first step of the cleavage reaction. Indeed, it is possible to amplify single-strand ends from hypermutating sequences (26), though whether the experiments in (26) detect one of the two ends generated by a DSB or alternatively, the unique end of a single-strand break remains unclear. Thus, regardless of the particular mechanics which lead to the creation of the DSB, the end product of the cleavage step of the reaction and hence, the substrate upon which DNA repair must act, in all three cases is likely to be a DNA DSB.

In vertebrates, the two main pathways of DSB repair are homologous recombination and nonhomologous end joining (NHEJ). These appear to operate predominantly in distinct phases of the cell cycle: G_1/early S for NHEJ and late S/G_2 for homologous recombination (27). The DSBs associated with V(D)J recombination are repaired by NHEJ and are found almost exclusively in G_1 phase cells. There is evidence to suggest that NHEJ might be the repair mechanism of choice for CSR-generated ends (25). In contrast, SHM-associated DSBs are found in G_2 phase cells and it has been postulated that they are repaired by homologous recombination (8, 28). Yet, while SHM and CSR have been proposed to involve distinct repair pathways, the end products of CSR (the switch joints) incur point mutations at high frequency (29) suggesting either that a common error-prone polymerase is involved in class-switch–associated NHEJ repair as well as in SHM-associated recombinational repair or that CSR repair requires both the NHEJ and the homologous recombination machinery.

Mechanistic overlap between CSR and SHM is further suggested by the finding that both reactions require AID (10). As ablation of AID leaves the targeting stage (the generation of germline transcripts) of CSR intact (10), AID has been proposed to function either at a pre or postcleavage step. In an attempt to distinguish whether AID is responsible for editing the nuclease or for coordinating repair, Nussenzweig and colleagues have looked at the association of repair factors with switch region DNA in wild-type or $AID^{-/-}$ mice (30). The repair factors Nbs1 and histone γH2AX are known to accumulate in high concentrations over areas which harbor DNA breaks, where they form distinct nuclear "foci." Such foci form readily over switch regions in LPS-IL-4–stimulated wild-type B cells which incur DNA breaks in the process of CSR, but are absent in similarly stimulated, AID-deficient B cells (presumably because of the lack of DSB formation in the absence of AID). Therefore in CSR, AID may act upstream of repair and perhaps functions in initiating DSB formation (30).

If SHM and CSR were two different repair outcomes of the similar DNA cleavage reactions as suggested previously (30), the expectation would be that DNA lesions would be absent in hypermutating sequences in AID-deficient cells. Our results, however, provide evidence that cells lacking AID, or expressing a dominant negative form of the protein, are still able to incur DNA lesions in SHM target sequences, which can be amplified and detected by ligation-mediated PCR. Ramos cells overexpressing AID DN, and germinal center B cells from $AID^{-/-}$ mice, incur DSBs in their Ig V regions without accumulating mutations (Fig. 3). Furthermore, Ramos cells expressing AID DN do not die, nor is their cell cycle perturbed, leading to the conclusion that they must repair the abundant DSBs efficiently and accurately (Fig. 2). Since $AID^{-/-}$ B cells are still able to incur DNA DSBs over hypermutating sequences, either AID plays different roles in SHM and CSR (for instance, by editing two separate mRNAs), or that DSBs are not mechanistically linked to the SHM reaction. There is little doubt that DSBs over hypermutating sequences are associated with the process; they only occur over sequences which hypermutate, they are extremely abundant, they coincide with mutational hotspots, and they are present in the phase of the cell cycle in which repair of SHM lesions probably occurs (10, 11, 13). However, it is possible that SHM is initiated by the introduction of single-strand nicks which get converted into mutations, and that the DSBs are formally not intermediates but rather byproducts of that reaction (18). Even then, the fact that DSBs are abundant over hypermutating sequences in AID-deficient cells argues strongly that the formation of the initial lesion (be it a nick or a DSB) as well as its potential byproducts is not dependent on the function of AID. Thus, though both CSR and SHM employ AID, they might do so in different steps of the two reactions.

We conclude that AID deficiency impairs SHM, but does not affect the creation of the DNA breaks associated with this process. Therefore, it is most likely that AID plays a role in the postcleavage (repair) phase of this reaction. Its action may transiently alter a DNA repair factor, thereby creating an error-prone repair machinery capable of resolving SHM DNA lesions. Our experiments further suggest that the cytidine deaminase catalytic core of AID is crucial for its role in SHM. Thus if AID is an RNA-editing deaminase, its role in SHM is most likely to edit the RNA of a DNA repair factor.

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